All-trans retionic acid stimulates IL-2-mediated proliferation of human T lymphocytes: Early induction of cyclin D3

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Summary

It has been known for many years that vitamin A protects man and animals from infectious diseases. Thus, vitamin A deficiency which is a problem in large parts of the world, leads to higher risk of infection-related deaths of children in theses areas. Supplementation of vitamin A has on the other hand been shown to protect against both infections and infection-related deaths, and this has been linked to overall stimulation of the immune system. However, despite numerous studies in man and animal models, it is still not clear how vitamin A stimulates the immune system. In our lab we have for several years studied the role of vitamin A on purified populations of B-and T-lymphocytes *in vitro*. We have found that proliferation of both human and murine B-cell precursors is inhibited by physiological levels of the vitamin A metabolite retinoic acid (RA) (Blomhoff et al., 1992; Fahlman et al., 1995; Naderi and Blomhoff, 1999). Also naive human B-cells are inhibited by RA (Fahlman et al., 1995), whereas the apoptosis in these cells is prevented (Lomo et al., 1998). In contrast to the effects on B-cells, we have shown that the proliferation of normal human T-lymphocytes is stimulated by RA (Ertesvag et al., 2002) and that also in T-cells, the apoptosis is prevented (Engedal et al., 2004). Furthermore, we have recently demonstrated that the effects of RA on both proliferation and apoptosis in T-cells, at least in part, are mediated via induced production of IL-2 (Engedal et al., 2004).

In this thesis we wished to elucidate if RA, independent of its ability to induce IL-2, also is able to enhance IL-2 mediated signaling in T-cells. First we documented that RA indeed was able to enhance IL-2 mediated proliferation. Thus, in the absence of serum, RA enhanced the proliferation of purified T-cells stimulated with anti-CD3 and saturating concentrations of IL-2, by a factor of 2-3. We next wished to determine how RA affected the cell cycle machinery that regulates G1-S-transition, and we found that RA first of all targeted cyclin D3. Thus, by enhancing the level of cyclin D3 at the mRNA and protein level, this resulted in increased phosphorylation of pRB, which is known to be the critical event in driving the cells into S-phase. Furthermore, we demonstrated that the effect of RA to enhance the expression of cyclin D3 leading to pRB phosphorylation and proliferation, was dependent on JAK. JAK-activation occurs downstream of the IL-2

receptor, and we found that the JAK-inhibitor AG-490 inhibited the effect of RA. Finally, we were able to show that the effect of RA on OKT3/IL-2 induced T-cell proliferation was dependent on the nuclear retinoid receptors RAR and RXR.

Abbreviations

ACAD	Activated T cell autonomous death
AICD	Activation-induced cell death
APC	Antigen presenting cell
APC	Anaphase-promoting complex
APS	Ammonium persulphate
Bis	N, N-methylene bisacrylamide
BH-domains	Bcl-2 homology domains
bp	Base pair
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumine
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CFSE	Carboxy-fluorescein diacetate, succinimedyl ester
CKI	CDK inhibitor
ConA	Concanavalin A
Cpm	Counts per minute
Cyt c	Cytocrom c
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DRB	5, 6-dichloro 1β-D-ribofuranosylbenzimidazole
EDTA	Ethylene diamine tetra acetic acid
ERK	Ectracellular regulated kinase
EtBr	Ethidium bromide
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein
G1	Gap 1 (in the cell cycle)
G2	Gap 2 (in the cell cycle)
GITC	Guanidine isothiocyanate
GSK	Glycogen synthase kinase
HCl	Hydrochloric acid
HEPES	N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]
HLA	Human Leukocyte antigen
HRP	Horse raddish peroxidase
IFA	Immunofluorescence assay-buffer
IFA-T	IFA-Tween
IgG	Immunoglobulin
IL	Interleukin
IL-2R	IL-2 receptor
ITAM	Immunoreceptor tyrosin-based activation motif
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LAT	Linker for Activation of T cells

MAPK	Mitogen-activated kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
mCi	mCievert
MHC	Major histocompatibility complex
MNC	Mononuclear cells
M-phase	Mitosis
NR	Nuclear receptors
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline solution
PDK1	Phosphoinositode-dependent protein kinase
PHA	Phytohemagglutinin
PH-domain	Pleckstrin homology domain
PI	Phosphoinositide
PI	Propidium iodide
PI3K	Phospho-inositol 3 kinase
PIP3	Phosphateidylinositol (3, 4, 5) Triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PMSF	Phenyl-methyl-sulphonyl fluorid
ppRB	Hyperphosphorylated RB
pRB	The retinoblastoma protein
PS	Penicillin and Streptomycin
PtdIns	Phosphateidylinositol
PTB	Phosphotyrosine-binding
PTK	Protein tyrosine kinase
RA	Retionic acid
RAR	Retionic Acid Receptor
RARE	Retionid acid response element
RNase	Ribonucleases
RNA	Ribonucleic acid
R-point	Restriction point
RXR	Retionid X Receptor
S	Synthesis
SDS-PAGE	SDS-polyacrylalaide gel electrophoresis
SEM	Standars error of the mean
SH2	Src homology 2
SH3	Src homology 3
STAT	Signal transducers and activators of transcription
tBid	Truncated Bid
TCR	T cell receptor
TNFα	Tumor necrosis factor
TPA	12-O-tetradecaanoylphorbol 13-acetate
TEMED	N, N, N', N'-tetramethylenediamine
UV	Ultraviolet

V	Volt
VAD	Vitamin A deficient
YXXM-motif	Tyr-X-X-Met-motif
Zap-70	Zeta Activating Protein

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1. INTRODUCTION

1.1 The cell cycle

The cell cycle is a highly ordered process which is defined as the period between two cell divisions (figure 1). In eukaryotic cells the cell cycle is divided into phases termed G1, S, G2 and M. G1 and G2 are Gap phases. G1 is the interval between mitosis (M phase) and DNA synthesis (S phase). During the G1 phase the cell integrates mitogenic and growth inhibitory signals, and makes the decision to proceed, pause or exit the cell cycle. S is the phase of DNA synthesis where the entire genome is replicated. After transition through the S phase, the cell enters the G2 phase where it prepares for the division process. The G2 phase is another point in the cell cycle, at which the cycle can be arrested, if for instance the DNA replication has not been completed or if DNA repair has to be performed because of DNA damage. During the M phase the replicated chromosomes are segregated into separate nuclei, and the cell divides into two daughter cells. G0 is a specialized resting state for cells that have excited the cell cycle, where they stay either temporarily or permanently (Alberts et al., 2002).

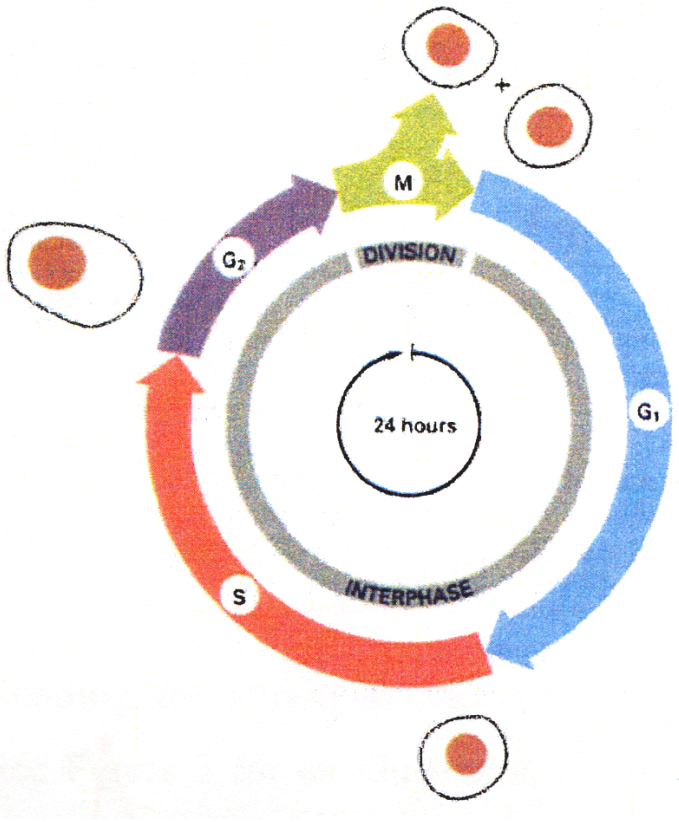


Figure 1. The four phases of the cell cycle (see text for details). During interphase the cell grows constantly, and during M phase the cell divides (Alberts et al., 1994).

1.1.1 The cell cycle machinery

i) General description

The cell cycle is controlled by different kinase complexes; each consists of a catalytic subunit, the cyclin dependent kinase (CDK), and a regulatory subunit, the cyclin. It is first when the CDKs bind to their specific cyclins that they become active, provided that these subunits are available (Pines, 1999). Although the levels of CDKs remain relatively constant throughout the cell cycle, cyclin abundance oscillates as a result of programmed synthesis and degradation through proteolysis (figure 2) (Coqueret, 2002; Ekholm and Reed, 2000). This ensures that the DNA is accurately replicated once per cell cycle, and that the chromosomes are segregated equally to daughter cells at the right time. The level of two classes of cyclins increases during the G1 phase of the cell cycle, D-type cyclins (cyclins D1, D2 and D3) and E-type cyclins (cyclins E1 and E2). D-type cyclins assemble with cdk6 or cdk4 as their catalytic partner in early/mid G1. Cyclin E mRNAs and

proteins begin to accumulate in late G1 and them associates with CDK2 (figure 3) (Coqueret, 2002; Ekholm and Reed, 2000). The cyclin E/CDK2 complex is required for the transition from G1 into S, and its activation can be regarded as the molecular "threshold" that has to be overcome for a cell to pass the restriction point (Planas-Silva and Weinberg, 1997), which is an important checkpoint in the mammalian cell cycle. Passage through the restriction point commits the cell to enter S phase. The level of cyclin A increases at the G1/S transition phase and it forms complex with CDK2. Late in S-phase cyclin A associates with CDK 1. It is suggested that cyclin A is needed for ongoing DNA replication in S phase, and thus for the completion of S phase, but it also plays an essential role in the initiation of mitosis. Cyclin A and cyclin B in association with CDK1 act synergistically in the initiation of mitosis, but once in mitosis, it is the B-type cyclins that seem to be the most important (Pines, 1995). Increased expression of both cyclins can be seen throughout mitosis. In order for the cell to exit mitosis, both cyclin A and cyclin B need to be degraded (Fung and Poon, 2005; McGowan, 2003).

The expression of D-type cyclins depends on continuous mitogenic stimulation, suggesting that D-type cyclins provide a link between mitogen signaling and the cell cycle machinery. Cyclin E, A and B expressions are mostly independent of extracellular signaling and are regulated at the transcriptional or post-transcriptional levels, like cyclin D (Coqueret, 2002). Figure 2 shows a typical pattern of cyclin expression during the cell cycle. The expressions of cyclin E, A, and B are periodically regulated throughout the cell cycle, whereas cyclin D expression is dependent on the presence of mitogen stimulation.

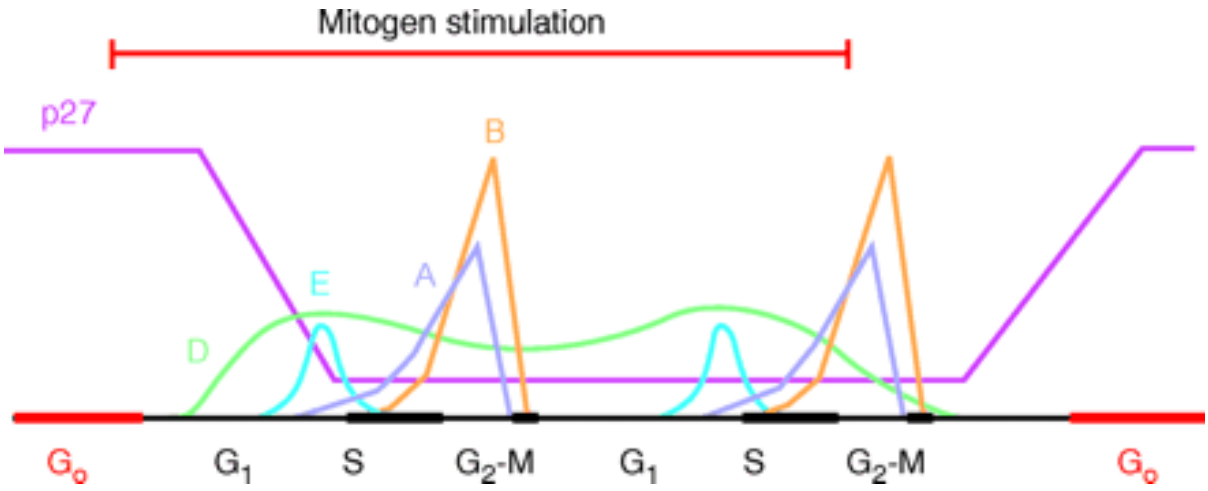


Figure 2. The fluctuations of cyclins and p27^{Kip1} during the cell cycle.
The expression of cyclins E, A and B is periodic, whereas D-type cyclins are expressed throughout the cycle in response to mitogenic stimulation. p27 levels are high in quiescent cells, fall in response to mitogen stimulation and remain low in proliferating cells (Sherr, 1996).

ii) Regulation of CDK activity

CDKs, either directly or indirectly, drive the cell-cycle transitions in all eukaryotic organisms. Due to their essential role in cell proliferation, they are subject to many levels of regulation in response to both intracellular and extracellular signals. This includes regulation of cyclin gene expression, post-translational modification of CDKs by phosphorylation-dephosporylation cascades, and by interaction with CDK inhibitors, the cip/kip-family of proteins (described in section 1.1.4). The first level of regulation is cyclin availability, and the fluctuation and regulation of these proteins is described in the previous section.

The unstable cyclin proteins are degraded via the ubiquitin/proteasome pathway. The polypeptide ubiquitin form conjugates with the cyclin proteins, signaling to the cells that the proteins are ready to be disassembled. This ubiquitination process is dependent on phosporylation of the cyclin (Ekholm and Reed, 2000). When ubiquitin is attached to the proper proteins, they are degraded by the 26S proteasome, a multicatalytic protease. There are two complexes, the Skp-Cullin-F-box (SCF) and the anaphase-promoting

complex (APC), and they target specific cell cycle components at different points in the cell cycle. The SCF complexes function at the end of G1, throughout S and in early G2, whereas the APCs become active at the end of G2 (Peters, 1998).

In addition to cyclin binding, the activity of CDKs is also regulated by phosphorylation and dephosporylation. In order to become active, cyclin/CDK-complexes are for instance phosphorylated on Thr 160 (in CDK2) by a CDK-activating kinase (CAK) (Morgan, 1997).Whereas cyclin binding is sufficient to activate CDK activity as mentioned previously, the Thr160 phosphorylation induces a further 80-300 fold increased activity, probably through improved substrate binding. Cyclin/CDK complexes are also inhibited by phosporylation at two sites (Thr 14 and Tyr 15 in cdk2). Wee1 is the major kinase responsible for phosphorylation of Tyr15 and Thr 14, whereas the Cdc25 phosphatase family (consisting of Cdc25A, Cdc25B and Cdc25C) reverse these inhibitory CDK phosphorylations (Coqueret, 2002; Ekholm and Reed, 2000; Russell and Nurse, 1987). Interestingly the cdc25 proteins are themselves substrates of the CDKs. Cdc25A for instance is phosporylated and activated by cyclin E-CDK2, suggesting that a positive feedback loop stimulates the rapid and full activation of CDKs.

iii) Cell cycle inhibitors

The activity of CDKs is regulated by the availability of cyclin subunits, the formation of cyclin/CDK-complexes, and by specific phosphorylation and dephosporylation (as described in section 1.1.1 ii). In addition, the activity of cdks in the different phases of the cell cycle is also governed by CDK inhibitors (CKIs). Two classes of CKIs has been identified, the Cip/Kip family and the Ink4 family.

Ink4 family

The Ink4 family is composed of four members: $p15^{Ink4b}$, $p16^{Ink4a}$, $p18^{Ink4c}$ and $p19^{Ink4d}$. They are so named because of their ability to only inhibit the catalytic subunits of CDK4 and CDK6 (Sherr and Roberts, 1999). The Ink4 inhibitors share a common structural motif and mechanism of inhibition. Ink4-bound CDKs cannot bind cyclin D (Ekholm and Reed, 2000).

Cip/Kip family

There are three members of the Cip/Kip family: $p21^{Waf1/Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$. They affect the activities of cyclin D-, E- and A-dependent kinases, and are able to bind both to cyclin- and CDK- subunits through a conserved N-terminal domain (Sherr and Roberts, 1999). The Cip/Kip proteins are potent inhibitors of cyclin E- and A-dependent CDK2, but they may act as positive regulators of cyclin D-dependent kinases (Sherr and Roberts, 1999). Due to their ability to bridge between cyclin and CDK (cyclinD/CDK4-complexes) at low concentrations, Cip/Kip inhibitors can serve as chaperonins, which facilitate the attainment of an active conformation, and they also increase the nuclear translocation and the stability of D cyclins (LaBaer et al., 1997). These findings indicate that Cip/Kip inhibitors may work as positive, as well as negative regulators of the cell cycle. However there exists conflicting data regarding the role of Cip/Kip inhibitors as positive regulators of cell cycle progression (Olashaw et al., 2004).

1.1.2 Cyclin D

As mentioned in section 1.1.1, D-type cyclins are the first to be expressed after mammalian cells are released from quiescence as a response to mitogenic stimulation. They bind to either CDK4 or CDK 6, and promote G1 progression by inactivating the growth suppressive properties of the retinoblastoma protein (see section 1.1.3). The raise of cyclin D levels seen in early G1 also serve to titrate Kip/Cip proteins away from cyclin E/CDK2 complexes, thus accelerating cell cycle progression (Coqueret, 2002). D-type cyclin genes have been shown to be transcriptionally induced by c-Myc (Perez-Roger et al., 1999), AP-1 (Bakiri et al., 2000) and NF-κβ (Guttridge et al., 1999) which are all

transcription factors. Cyclin D is also regulated at the translational level which includes the PI3K-pathway (Muise-Helmericks et al., 1998). Mitogens have the ability to increase the rate of i.e. cyclin D1 translation by activation of the translation initiation factor eIF-4E (Sonenberg and Gingras, 1998), while antiproliferative agents do decrease cyclin D1 synthesis through inactivation of the same factor (Aktas et al., 1998). During the G1-phase, cyclin D/CDK complexes assemble and get imported into the nucleus in a process which requires an active MAP-kinase pathway, and a molecular chaperone. During S phase D-type cyclin-CDK complexes are exported into the cytoplasm, and this is linked to the ubiquitin-mediated proteolysis (Pines, 1999).

i) Cyclin D3 and cyclin D2

In T-lymphocytes the major D-cyclins are D2 and D3, while D1 is not detected at all (Ajchenbaum et al., 1993). Cyclin D3 has been shown to be a key cyclin controlling T cell proliferation and apoptosis (Hleb et al., 2004). Among all D-type cyclin/CDK4- and CDK6 complexes, cyclin D3/CDK4 is most active in sequestering $p27^{Kip1}$ away from cyclin E/CDK2, thereby allowing progression through G1(Zhang et al., 2005). Furthermore, Lea and coworkers suggest that there is a commitment point during G0 → G1 that controls activation into the cell cycle in T cells, and that activation of cyclin D2-CDK6/4 is rate-limiting for progression through this point (Lea et al., 2003). They found that expression of cyclin D2 is induced by TCR/CD3-initiated signals, whereas cyclin D3 expression seems to be primarily induced by IL-2 signaling.

1.1.3 Regulation of the G1 to S transition (the RB pathway)

i) pRB-dependent regulation of S-phase entry

The transition through the different phases of the cell cycle is controlled by phosphorylation of specific targets of the cyclin/CDK-complexes. One of the best studied substrates of the G1 cyclin/CDK-complexes is the retinoblastoma protein (pRB), which is a member of the family of "pocket proteins". The retinoblastoma gene (RB-1) was identified over a decade ago as the first tumor suppressor. The gene was initially cloned as a result of its frequent mutation in the rare pediatric eye tumor, retinoblastoma, where

a loss or mutation is seen in both alleles of the Rb-1 gene. Now it is thought to play an essential role in cellular regulation, where it has a key role in controlling the so called "restriction (R) point" in the G1 phase of the cell cycle (Harbour and Dean, 2000b; Hu et al., 1990). The restriction point, which occurs in mid-late G1 phase, subdivides G1 into two phases-early/mid G1 and late G1 phase (Zetterberg et al., 1995). The cells respond to extracellular signals in the period before the restriction point, and in the remainder of the cell cycle they are relatively, if not totally ignorant to such signals; thus once the cells have passed the R point, they seem to be committed to complete the remainder of their cell cycle program in an essentially automatic fashion (Planas-Silva and Weinberg, 1997).

The pRB primary structure is subdivided into several domains. The pocket region is composed of two highly conserved regions, A and B, which interact with each other along an extended interdomain interface which forms the central "pocket". The A-box portion seems to be required for the stable folding of the B-box (Harbour and Dean, 2000b; Lee et al., 1998). The A and B domains form a transcriptional repressor motif, and their protein binding capacity is regulated by cyclin dependent kinases (Muller and Helin, 2000). Mutations affecting the retinoblastoma gene are frequently encountered in various human cancer forms (Harbour and Dean, 2000b; Horowitz et al., 1989).

pRB function depends, at least in part, on interactions with the E2F family of transcription factors (Harbour and Dean, 2000b). E2F regulates the expression of several genes that encode proteins involved in cell cycle progression and DNA synthesis, such as cdc25, cyclin E and A and DNA polymerase α. E2F transcription factors can appear in free form as heterodimers containing a subunit encoded by the E2F gene family, and a subunit encoded by the DP family of genes. Alternatively it can form complexes containing E2F/DP heterodimers that are stably bound to a member of the pRB family of proteins (Dyson, 1998).

The G1/S transition is regulated by the phosphorylation state of pRB. In quiescent cells, pRB is in a hypophosphorylated state, and it is this form of pRB that binds and inhibits

E2F, thus blocking the restriction point transition (Harbour and Dean, 2000b). The phosporylation of pRB is initiated by complexes containing D-type cyclins and either CDK4 or CDK6 in mid/early-G1 phase (figure 3). These complexes only initiate pRB phosphorylation, and do not drive it to completion. That seems to be accomplished by the CDK2-cyclin E complexes which are activated just before the cell passes through the R-point and phosporylates pRB on additional sites. RB hyperphosporylation in late G1 disrupts its association with various E2F family members, and this allows the coordinated transcription of several genes whose activitities are necessary for DNA synthesis (Coqueret, 2002; Harbour and Dean, 2000b; Hatakeyama et al., 1994; Resnitzky and Reed, 1995).

Among the E2F-regulated genes are cyclins E and A, which both are required for the G1/S transition in normal cells. The capability of E2F to induce cyclin E, which in turn regulates CDK2 to maintain pRB phosporylation, creates a positive feedback loop that helps add to the irreversibility of the G1/S transition (Sherr and Roberts, 1999). Cyclin A- and B-dependent CDKs which become activated later during the cell cycle maintain RB in a hyperphosporylated form until the cells exit mitosis and RB is returned to a hypophosphorylated state in the next G1 phase (Sherr and Roberts, 1999).

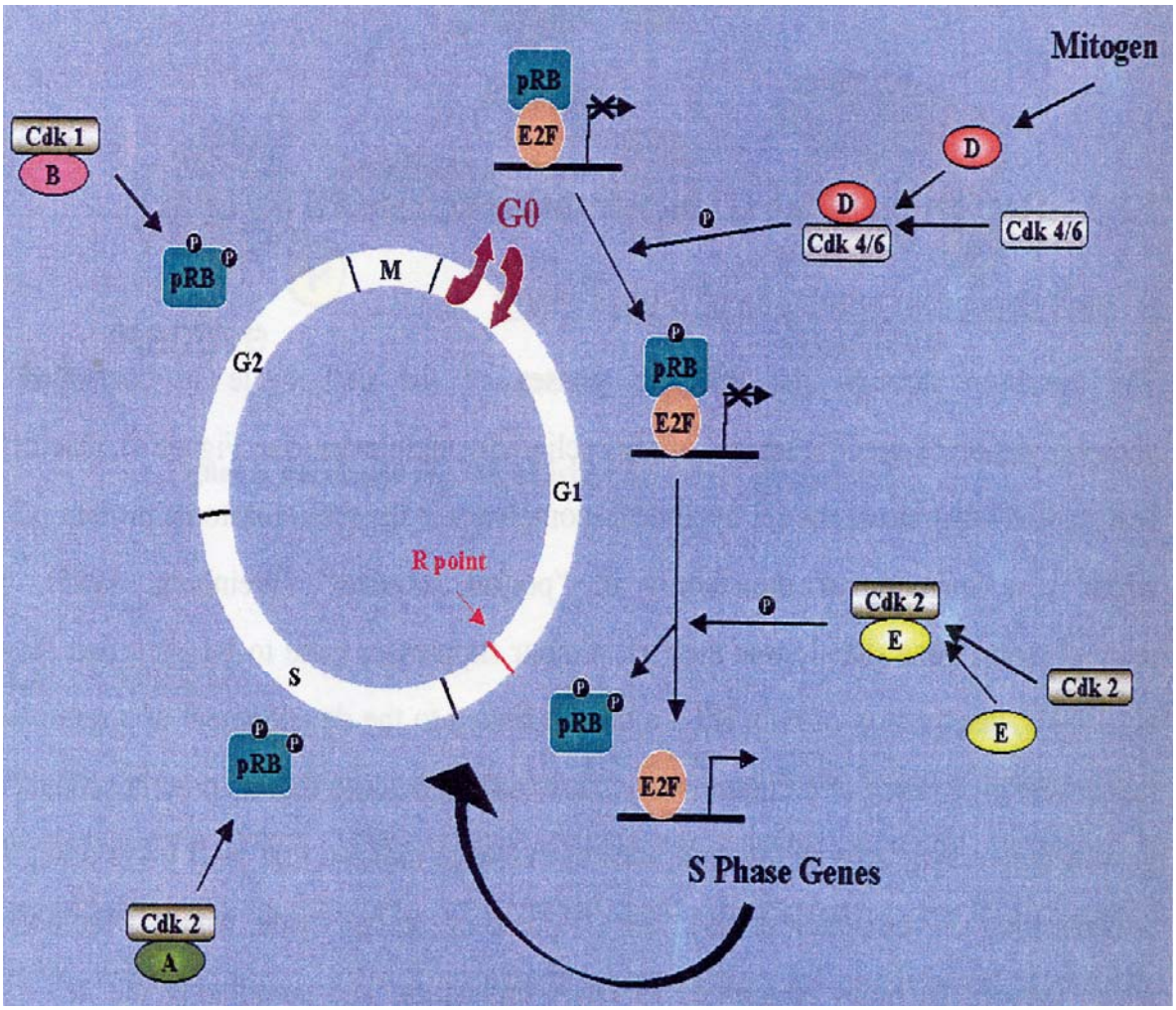


Figure 3. Schematic illustration of the cell cycle machinery (see text for explanation).
G0, G1, S, G2 and M refer to the quiescence, first gap, DNA synthesis, second gap and mitosis of the cell cycle, respectively. R point indicates the restriction point in G1. D, E, A, B and P indicate cyclin D, cyclin E, cyclin A, cyclin B and phosphate group, respectively.

ii) pRB-independent regulation of S-phase entry

Evidence suggests that cyclin E may be able to promote S phase entry via pathways that do not involve pRB. Lukas and co-workers have demonstrated that cyclin E can induce S-phase without activation of the pRB/E2F pathway, and that cyclin E can act downstream from Rb/E2F, suggesting that there are additional substrates for cyclin/CDK2 complexes (Lukas et al., 1997). Furthermore, expression of cyclin E or transcriptionally active Myc

can rapidly induce DNA synthesis in cell lines where E2F activity is blocked by expression of constitutively active pRB mutant. The effect of Myc requires both cdc25A phosphatase (mentioned in section 1.1.1ii) and cyclin E, both known transcriptional targets of Myc (Santoni-Rugiu et al., 2000). These results suggest that cyclin E also is a target of a G1/S-promoting mechanism different from the pRB pathway, where Myc is involved.

1.2 T lymphocytes

The immune system comprises cells in blood, lymph, bone marrow, thymus, spleen and lymph glands. Broadly outlined, the immune system can be divided into two parts; i) non-adaptiv/ innate immunity and ii) adaptive immunity. Innate immunity provides the first line of defense against foreign agents, and is not dependent of previous exposure to antigens. While cells in the innate immune system lack specificity against the foreign "invaders", cells in adaptive immunity are characterized by having both immunologic specificity and immunological memory. Lymphocytes, i.e. T cells and B cells, constitute the adaptive immune system. The cellular part of the adaptive immune response is mediated by T cells, whereas the humoral immune response is mediated by B cells which produce specific antibodies against foreign agents.

T cells are derived from bone marrow stem cells. Immature precursor T cells leave the bone marrow and migrate to the thymus. Here they undergo gene rearrangement which produces cells with unique antigen receptor expression and develop into mature T cells capable of responding to foreign antigens. In thymus only thymocytes with weak specificity for "self" Human Leukocyte Antigen (HLA)-molecules survive the first positive selection process. Thus, thymocytes that react too strong with "self" HLA-molecules are killed by apoptosis during a process called negative selection (Lea, 2000).

1.2.1 TCR/CD3-signaling

It is well known that T cells need two signals in order for them to be activated and proliferate. First, antigenic stimulation of the TCR/CD3 complex drives the cells from

their quiescent state (G0) into the G1 phase of the cell cycle (Lea et al., 2003). Second, the binding of IL-2 to its high affinity receptor leads to G1-to S-phase transition and thus proliferation (Nelson and Willerford, 1998).

The T cell antigen receptor (TCR) is triggered in response to foreign peptides presented on molecules encoded within the major histocompatibilty complex (MHC) on antigen-presenting cells (APCs) (Schrum et al., 2003). The T cell receptor (TCR) consists of two heterodimeric αβ or γδ polypeptide chains. However, most T cells (in mice and humans) express αβ TCRs (Schrum et al., 2003). The TCR is tightly linked to a CD3 complex which consists of three invariant polypeptides γ, δ and ε and two additional homodimeric (ζ- ζ) or heterodimeric (ζ-η) subunits. This CD3 complex transduces the activation signal generated by ligand binding to TCR (Mustelin and Tasken, 2003).

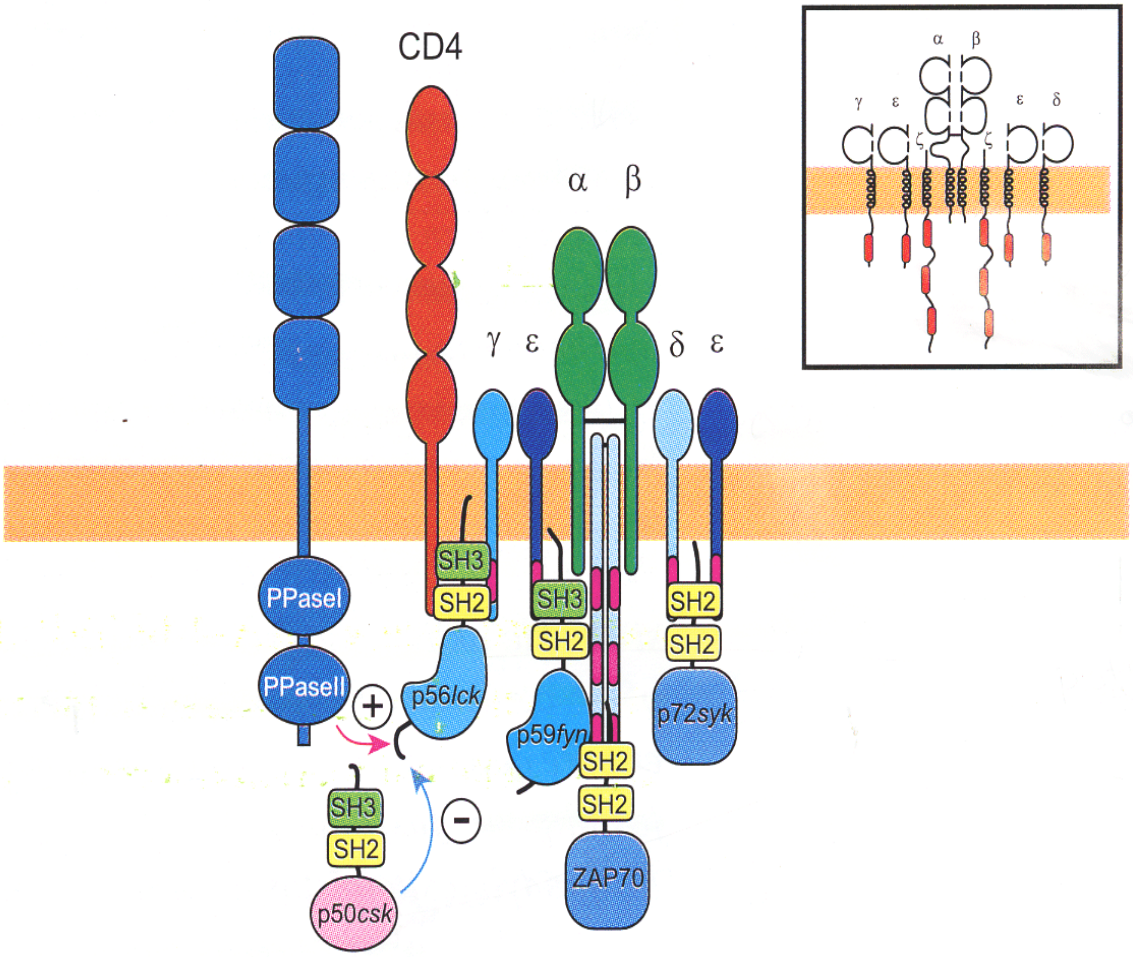


Figure 4. The schematic illustration of the mechanism behind the TCR/CD3-complex-mediated activation of the cell (see text for details). The ITAM-sequences are marked in red (Lea, 2000).

There are also other accessory molecules that are involved in T-cell activation. The CD4- and CD8-co-activators are central in T- lymphocytes activation. $CD4^+$ T-cells only react with antigens bound to HLA-class II, whereas $CD8^+$ T-cells recognize antigens in complex with HLA-class I-molecules. The binding between the CD4/CD8- and HLA-molecules are relatively weak, but strong enough to have an important functional influence, because they both are associated with $p56^{lck}$, a protein tyrosine kinase, which will be discussed later. CD28 is another important interaction molecule involved in the activation of T-cells. It has two ligands, B7.1 (CD80) and B7.2 (CD86), presented on the membrane of APCs. When a T cell with its TCR binds to an APC with B7-molecules on

the surface, then the binding between B7 and CD28 will result in signal 2. This signal is necessary in order for T cells to proliferate and mature into effector cells, which will be described in section 1.2.2 (Lea, 2000).

A key initiating event in T cell activation is the increased phosphorylation of immunoreceptor tyrosin-based activation motif (ITAM) tyrosines in the TCR subunits. These ITAMs are found mainly in the Src family tyrosine kinases $p56^{lck}$, but also $p59^{fyn}$ (Mustelin and Tasken, 2003). Phosphorylation of the ζ chain recruits the tyrosin kinaseZap-70(Zeta Activating Protein), but subsequent phosphorylation of Zap-70 by Lck is needed to activate the kinase (see figure 5). Once activated, Zap-70 phosphorylates a membrane bound adapter protein, LAT (Linker for Activation of T-cells). LAT recruits several proteins, for instance downstream Src homology 2(SH2) and SH3 domain-containing proteins. The recruitment of these proteins activates various signalling pathways which end up in the nucleus and result in activation of transcription factors which for instance regulate the expression of the gene for IL-2 (IL-2) (Lea, 2000).

1.2.2 IL-2R and IL-2 signaling

The activation of CD4-+ T cell by antigen makes it increase in size, and it becomes a so-called blast cell. About six to eight hours after the activation, changes in cell surface molecules can be shown. A new activation antigen, CD25, the important α- polypeptide chain for the high-affinity receptor IL-2, emerges. After about twelve hours, activated T-cells produce and secrete IL-2, which will be bound to the IL-2 receptor (IL-2R). The binding of IL-2 to the IL-2R will result in CD4-+ T cell proliferation (Lea, 2000).

The high-affinity IL-2 receptor comprises three subunits, the α, β and γ-chains (Gesbert et al., 1998). Resting T cells generally express low to intermediate levels of the β and γ subunits and no, or very low, levels of IL-2R- α on their cell surface. Upon T cell activation, the cell surface levels of IL-2R- α are rapidly increased, while the surface levels of the β and γ subunits are only moderately increased. Both the α- and β-chain can

bind to IL-2, but only with low affinity. However, physiological IL-2 signaling is believed to occur through binding of IL-2 to the high affinity, heterodimeric (αβγ) IL-2 receptor (Lea, 2000). The α-chain is responsible for IL-2 binding and internalization, and does not appear to be involved in intracellular signaling (Gesbert et al., 1998).

None of the chains in the IL-2 receptor complex have enzymatic activity, so the activation signals are transmitted into the cell by recruiting protein tyrosine kinases (the PTK Syk of the Syk/ ZAP family, and Jak1 and JAK3 of the Janus kinase family) to the complex (Gesbert et al., 1998). In the presence of IL-2, the three subunits of the IL-2 receptor are brought together (see figure 5). Thereby, tyrosine kinases, such as Janus kinase 1 (JAK1) and Janus kinase 3 (JAK3), which associate with β and γ subunits, respectively, are also brought together, and are thought to activate each other through reciprocal phosporylation (Lea, 2000).

JAK activation results in the phosphorylation of several tyrosine residues on the β subunit, which serve as docking sites for signaling molecules. There are for instance two independent pathways that are initiated from these docking sites, and which are believed to play major roles in IL-2-mediated proliferation and survival. These are the JAK/STAT pathway and the MAPK/ERK pathway (Ellery and Nicholls, 2002; Gesbert et al., 1998; Lin and Leonard, 1997). Furthermore, the PI3K-pathway is another signaling pathway downstream for the IL-2 receptor.

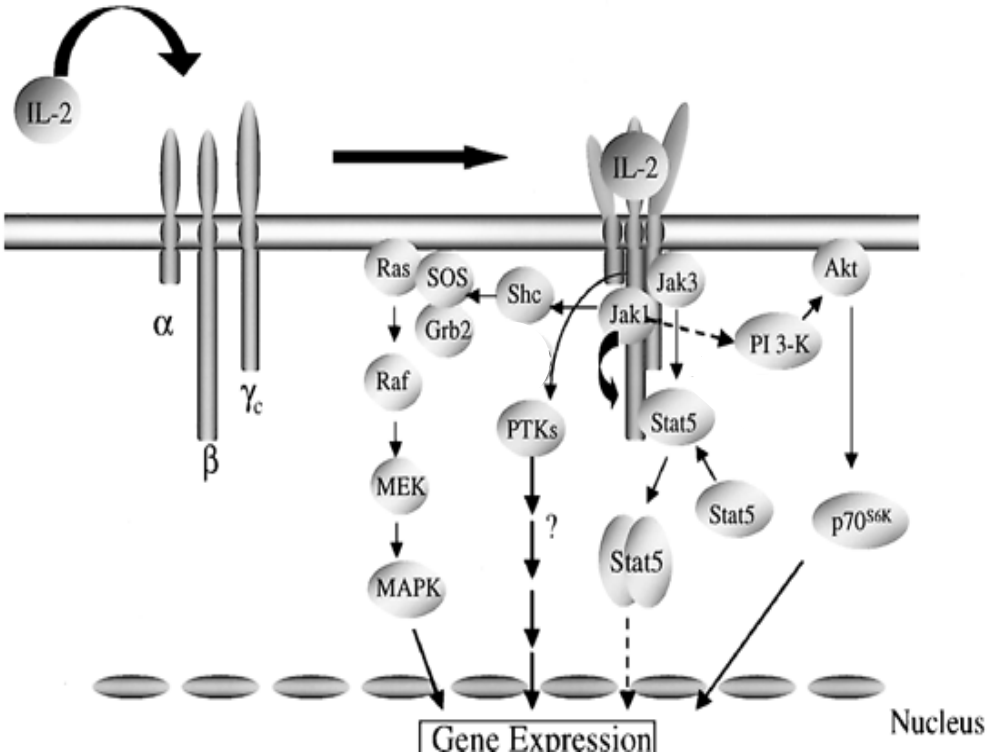


Figure 5. Schematic illustration of the signaling pathways associated with IL-2R β and /or γ-subunit which are known to be activated by IL-2. PTKs represent the various protein tyrosine kinases mentioned in the text. The broken lines and question mark indicate signaling pathways not fully understood, and they are therefore not described further in the text. The figure is modified from Lin and Leonard, 2000.

i) <u>The JAK/STAT pathway and the MAPK/ERK pathway</u>

Activation of the JAK/STAT-pathway results from docking of STAT5 to Y392 and Y510 of the β subunit (Lin and Leonard, 1997). After docking, this transcription factor is phosphorylated by JAKs, which results in their homodimerization and activation. The STAT5 homodimer translocates to the nucleus, where they will regulate transcription of genes which are necessary for proliferation and differentiation of T cells to effector cells (see figure 5) (Benczik and Gaffen, 2004; Gesbert et al., 1998).

In general, the MAPK/ERK pathway takes part in cell proliferation and inhibition of apoptosis (Chang et al., 2003).

The adaptor protein Shc is first docked to Y338 of IL-2R-β (Lin and Leonard, 1997). Shc recruits proteins that activate the Ras-Raf-MAP- and PI3-Akt-kinase pathways (see next section), respectively. Then, the formation of a Shc-Grb-Sos trimer occurs. Shc and Grb2 connect the receptor to Son of Sevenless (sos), a guanine nucleotide exchange factor, which in turn tranduces a signal to Ras, a small GTP binding protein. Thereafter three signaling molecules are activated: Raf (MAPKKK), mitogen activated protein kinase kinase (MEK) 1/2 (MAPKK) and MAPK1/2 (see figure 5). The phosphorylated ERK dimer can regulate targets in the cytosol and the nucleus by activating other protein kinases such as ribosomal S6 kinases and Mnk, which in turn phosporylate proteins involved in chromatin remodeling, or transcriptions factors such as for example c-Jun, c-Fos and c-Myc (Benczik and Gaffen, 2004).

In addition to the STAT5 and Shc pathways, activation of another STAT transcription factor, STAT3, may also be involved in IL-2-mediated proliferation (Ellery and Nicholls, 2002). STAT3, which is constitutively associated with IL-2R-β, is also tyrosine phosporylated by JAKs. Later it is phosphorylated on a serine residue by ectracellular regulated kinase (ERK). Both phosphorylation events are essential for maximal STAT3 transcriptional activity (Wen et al., 1995).

ii) PI3K pathway

The PI3K pathway is so far the best characterized pathway downstream of the IL-2R. The phosphateidylinositol-3-kinase is a family of lipid and serine/threonine kinases. Phosphatidylinositol (PtdIns) consists of a glycerol backbone to which two fatty acids and an inositol 1-phosphate group are attached (Figure 8). A phosphorylated PtdIns is called a phosphoinositide (PI). All PIs reside in membranes, where they interact with a lot of cellular proteins via unique lipid-binding motifs, such as pleckstrin homology (PH)

domains, Src homology-2 (SH2) domains and phospotyrosine-binding (PTB) domains (Rameh et al., 1997; Rameh and Cantley, 1999).

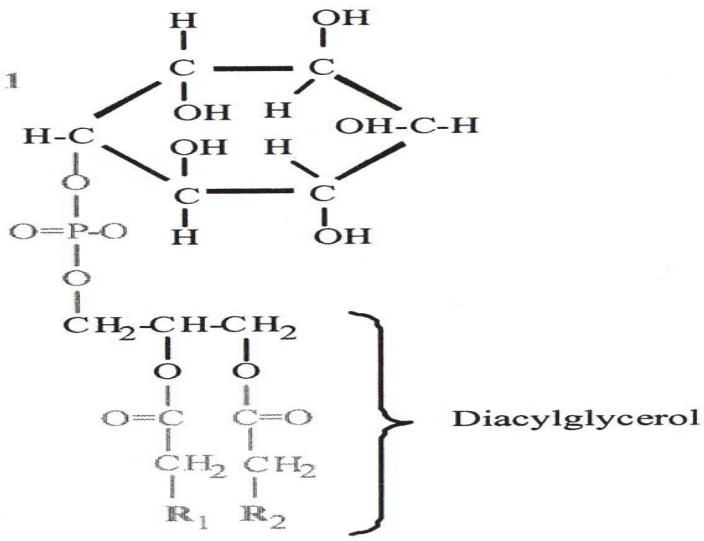


Figure 6. Chemical structure of phosphatidylinositol. The inositol head group is localized in the cytosol, whereas the fatty acid tails (R1 and R2) lie within the inner leaflet of lipid bilayer.

PI3K family members are divided into four classes (class Ia, Ib, II, III) based on sequence homology and substrate specificity, but it is mainly the PI3K molecules from class Ia that become activated in response to cytokine signaling. Class Ia PI3Ks are heterodimers which are composed of two subunits: a catalytic subunit with a molecular weight of 110 kD (p110α, p110β or p110δ) and a regulatory subunit (p85α, p55α, p50α, p85β, or p55γ). The activation of class Ia PI3K is induced when tyrosine kinases phosporylate membrane proteins, and receptors at residues that are located within a special Tyr-X-X-Met (YXXM) motif. This in turn serves as a docking site for the SH2 domain of a regulatory subunit of PI3K, thus bringing the catalytic subunit in proximity with the membrane and its lipid substrates. This step then activates the p110 kinase, which is otherwise inhibited in the p85-p110 complex (Benczik and Gaffen, 2004).

PI3K can connect with the IL-2R complex via Shc-Grb2-Gab2, and IL-2 stimulation of T cells results in a rapid activation of PI3K and phosphatidylinositol (3,4,5) triphosphate (PIP3) (Benczik and Gaffen, 2004).

Downstream of PI3K, the protein kinase Akt/PKB is recruited to the membrane-anchored PIP_2 and PIP_3 (lipid substrates of PI3K). To be fully active, Akt/PKB needs to be phosporylated at two sites (Ser 473 and Thr 308). PDK 1 (phosphoinositode-dependent protein kinase) is the upstream kinase that targets Thr 308 (Benczik and Gaffen, 2004), whereas the kinase responsible for phosphorylation of Ser 473 remains unidentified.

1.2.3 Regulation of the T cell cycle machinery by IL-2/IL-2R

TCR-CD3 signals drive resting T cells from their quiescent, G0 state, into the cell cycle (Firpo et al., 1994). However, in the absence of IL-2, S phase entry does normally not occur (Firpo et al., 1994; Modiano et al., 1995). Thus, the CDK2 complexes are not generated, and the cells are arrested in G1, due to the inability of TCR/CD3 signals to down-regulate the expression of $p27^{Kip1}$. In the presence of IL-2, will $p27^{Kip1}$ protein levels become strongly downregulated, CDK2 becomes active, and the cells are driven into S phase (Firpo et al., 1994; Kwon et al., 1997). Interestingly, the protein levels of another CKI, $p21^{Cip1}$, are increased by IL-2 (Nourse et al, 1994; Firpo et al, 1994), which can be explained by the dual role of p21 as both a CDK activator and inhibitor (see section 1.1.1, iii).

1.3 Cell death

1.3.1 Apoptosis and necrosis

In general, cells die through one of two alternative pathways: i) apoptosis, a programmed and highly ordered form of cellular destruction, or ii) necrosis, a passive and unregulated form of cell death. Apoptosis is an active process which requires protein synthesis for its execution. Characteristic morphological features like cell shrinkage are seen in cells

which undergo apoptosis (Walker et al., 1988). The most evident changes occur in the nucleus, which includes chromatin condensation and nuclear shrinkage, as seen in figure 7 (Walker et al., 1988). A characteristic feature is also the formation of apoptotic bodies (Walker et al., 1988). The morphological changes which appear are a result of molecular alterations, such as DNA and RNA cleavage, post-translational modifications of nuclear proteins, and proteolysis of several polypeptides residing in the nucleus (Martelli et al., 2001).

A hallmark of apoptosis is the cleavage of DNA into 200 bp fragments, which can be detected either by electrophoreses, or by flowcytometric analysis using the TUNEL method. Apoptosis is vital for differentiation, morphogenesis, development and homeostasis of multicellular organisms, and it is the most usual form of physiological cell death. Apoptosis plays a major role for the maturation of the immune system, and can therefore be involved in disorders that origin in the immune system, for example AIDS where mature $CD4^+$-cells dies, and autoimmune disease (Martelli et al., 2001). For instance, genetic analysis indicates that deficiencies in serum proteins or receptors that mediate clearance of apoptotic cells increase the risk of autoimmunity (Cline and Radic, 2004).

In mammals, diverse death stimuli induce apoptosis by activating the caspase family of cyctein proteases (Breckenridge and Xue, 2004). The caspases are activated in a cascade-ordered manner by two different main pathways, one being mediated via death receptors, and the other process inducing mitochondria (Breckenridge and Xue, 2004; Green, 2005; Kroemer and Martin, 2005). Caspases are proteolytic enzymes, and when inactive they lay dormant in the cell as zymogens.
It is generally believed that the Bcl-2 family of proteins and sometimes the caspases themselves governs the release of apoptogenic factors from the mitochondria. Bcl-2 is a proto-oncogene; the normal cellular gene from which an oncogene has been derived. Bcl-2 family proteins can be subdivided into three main classes on the basis of their functions and the number of Bcl-2 homology (BH) domains present. Class 1 is antiapoptotic and contains four BH domains (BH1 to BH4). Bcl-2 and Bcl-xL belong to this group. Bak

and Bax are members of the class 2 proteins which are pro-apoptotic and they possess three BH domains (BH1-BH3). The BH3-only pro-apoptotic members such as Bid and Bim share homology only within the BH3 domain, and they belong to the third class (Breckenridge and Xue, 2004).

It is generally accepted that distinct apoptotic signals first converge upon different BH-3-only proteins, which upon activation deliver the death signals to mitochondria by engaging Bax/Bak or Bcl-2/Bcl-xL. BH-3-only proteins are activated for instance by post-translational modifications, such as caspase-8 mediated cleavage of Bid into an activated, truncated Bid (tBid). Bax and Bak mediate permeabilization by forming pores in mitochondria. The formation of tBid amplifies initiator caspase signals by inducing the release of proteins such as cytocrom c (cyt c) (Breckenridge and Xue, 2004). When cyt c is released from mitochondria, it interacts with APAF-1 and procaspase-9. This complex favors the activation of caspase-9 which, when activated, cleaves and activates caspase-3 which in turn cleaves other caspases. These molecules then cleave substrates in the cell to produce the cellular and biochemical events we see in apoptosis (Green, 2005).

It is suggested that Bcl-2 anti-apoptotic proteins may function both by sequestering active BH-3-only proteins and by restricting Bax/Bak oligomerization, thus setting an activation barrier for the induction of apoptosis and limiting inappropriate cell death. During apoptosis, the anti-apoptotic activity of Bcl-2 is probably overcome by simultaneous activation of distinct classes of BH3-only proteins, some of which bind to and inactivate Bcl-2. This sets BH-3-only proteins free to activate Bax/Bak (Breckenridge and Xue, 2004; Green, 2005).

Necrosis is another type of cell death, and it is a result of irreparable cell injury, often caused by stimuli that damage cell membranes or inhibit energy production in the cell (Endresen and Aarbakke, 1992). Necrosis is characterized by swelling of the cell, and when the cells burst, this leads to lysis of the cells (see figure 7). The cell content which leaks out extra-cellularly causes inflammation. Random DNA degradation, and not DNA fragmentation as in apoptosis, is observed in necrotic cells (Walker et al., 1988). There is

increasing awareness that these two pathways, apoptosis and necrosis only represent the extreme ends of a variety of possible morphological and biochemical deaths (Martelli et al., 2001).

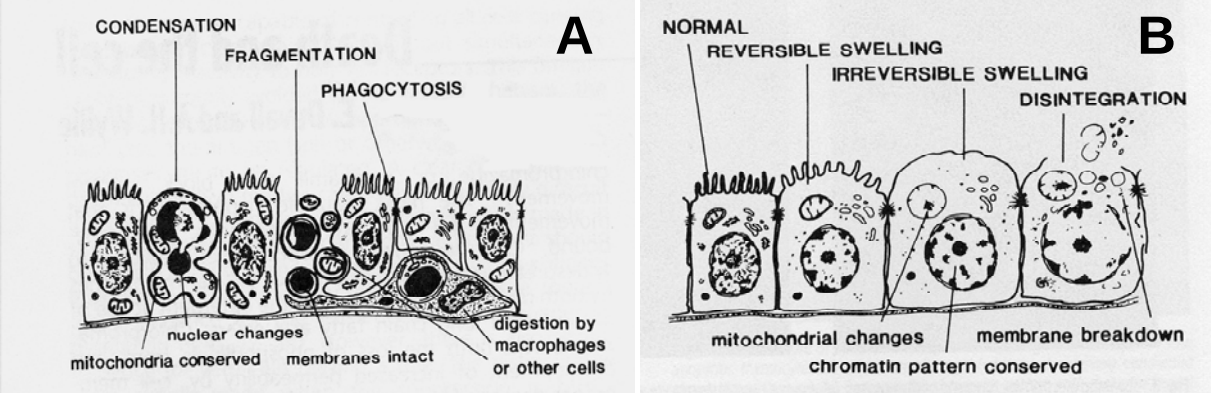


Figure 7. The morphology of necrosis and apoptosis. The typical morphological features of apoptosis (A) and necrosis (B) are shown. Adapted from Duvall and Wyllie, 1986.

1.3.2 T cell death: AICD and ACAD

Resting T cells are reasonably resistance to apoptosis, partly due to their high expression of anti-apoptotic Bcl- 2 proteins (Salmon et al., 1994). T cells respond to antigen-stimulation through a process of activation, division and differentiation resulting in a rapid accumulation of a large amount of activated effector T cells. Once T cells are activated, they become much more susceptible to die. This is logical, since resting T cells need to stay alive for a long time in order to maintain the body`s repertoire of antigen-scanning. Activated T cells, however, need to die at some point; either because they are not needed, as when the immune system “shuts” down after the antigen has been cleared, or because they are continuously activated by self-antigens and therefore should be deleted. Following a peak of expansion, their numbers begin to decline rapidly through a process termed the contraction phase. During this decline some of the T cells migrate to nonlymhoid tissues, where they reside as memory T cells, but most of the activated T cells disappear through apoptosis (Hildeman et al., 2003).

Two distinct pathways have been shown to be responsible for the death of activated T cells in vivo: activation-induced cell death (AICD) and activated T cell autonomous death (ACAD) (Hildeman et al., 2002; Hildeman et al., 2003). AICD is initiated by the activation of death receptors; Fas, but also by TNFα, when T cells encounter repeated self-antigen. Once these receptors are engaged, they activate downstream pathways that lead to caspase activation and cell apoptosis. In contrast, ACAD proceeds after a single exposure to antigen (during the termination of an immune response) through a mitochondrial pathway and is driven by intrinsic (i.e. without the involvement of other cells) signals in the activated T cell that are independent of death receptors to induce apoptosis (Hildeman et al., 2002; Hildeman et al., 2003). This cell death is initiated by an activation-induced alteration in the balance between pro- and anti-apoptotic members of the Bcl2 family, which leads to mitochondria-induced apoptosis. ACAD is responsible for the death of the majority of T cells which responds to foreign antigen (Hildeman et al., 2002).

1.4 Vitamin A

1.4.1 Vitamin A and its physiological role

Vitamin A is defined as substances with the same biological effect as retinol. Vitamin A (retinol and its derivatives) is involved in various biological processes, such as growth, reproduction, cellular differentiation, embryonic development and visual response. Vitamin A is also imortant for normal immune function, which will be discussed in a later section (see section 1.4.3).

Vitamin A is a nutrient and derives from carotinoids in plants and retinyl esters in animal tissues. Under normal dietary conditions vitamin A is present at relatively high concentrations (3μM) in plasma and therefore potentially available to all cells in the body (Blomhoff et al., 1990). The major source of retionids for target cells in vivo is retinol bound to retinol-binding protein or retinylester in chylomicron particles (Blomhoff, 1994). However, inside target cells retinol can also be oxidized to the active metabolite

retinoic acid (RA). Several naturally isoforms of RA has been detected, which includes all-trans RA and 9-cis RA. They are the most active retionids in most cell types.

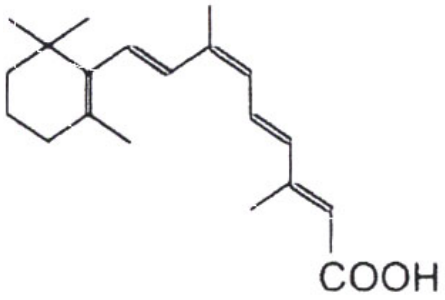


Figure 8. Structure of 9-cis retinoic acid

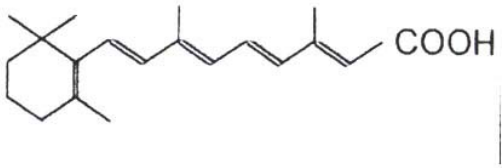


Figure 9. Structure of all-trans retinoic acid

1.4.2 General mechanism of action of vitamin A

Vitamin A and its metabolites are signaling molecules that act through interaction with two families of retionid receptors, retinoic acid (RAR α, β and γ) and retionid X (RXR α, β and γ) receptors. These receptors belong to the superfamily of nuclear receptors (NRs), including various receptors as those for steroids and thyroid hormones, and vitamin D3. The NRs act as ligand-inducible transcription factors (Escriva et al., 1997).

RARs and RXRs form heterodimers, whereas RXRs form homodimers, as well as heterodimers with other members of the nuclear receptor family. RXRs may for example form heterodimers with vitamin D receptors, thyroid receptors and peroxisome proliferators-activated receptors (PPAR) (Kliewer et al., 1992).

atRA preferentially binds to and thereby activates RARs, whereas 9-cis RA activates both RARs and RXRs. RXRs bind and are activated by 9-cis RA with K_d values of 1.4 to 2.4 nM, and RARs activate 9-cis RA with K_d values of 0.2-0.7 nM (Roy et al., 1995). RAR-, but not RXR-ligation is sufficient to activate RAR/RXR heterodimer. In addition, simultaneous ligation of both RAR and RXR acts synergistically on RAR/RXR transcriptional activity (Bastien and Rochette-Egly, 2004; Gronemeyer and Miturski,

2001). More than 100 genes have been found to be directly regulated by RA-mediated activation of RAR/RXR. Furthermore, RA can indirectly regulate the expression of several hundred other genes (Balmer and Blomhoff, 2002). Although, the RAR/RXR-pathway has been shown to be the major signaling pathway, other pathways might also be included. First, RXR/RXR may regulate genes, but this has not been proven in vivo (Mangelsdorf et al., 1991). Second, although RXR usually acts as a "silent" partner with other nuclear receptors than RAR (mentioned above), it may act as a ligand-dependent transcriptional regulator in some circumstances (Leblanc and Stunnenberg, 1995). Third, RA may also function independent of RAR/RXR (Konta et al., 2001; Radominska-Pandya et al., 2000). Konta and coworkers examined the role of at-RA in apoptosis and whether RAR and RXR are required for the at-RA mediated suppression of c-fos/c-jun expression and c-Jun N-terminal kinase (JNK). They demonstrated that suppression of JNK activation by at-RA was mediated by neither RAR nor RXR. The authors suggest that the suppressive effect of at-RA in this study might be mediated by other types of receptors. For instance, it has been reported that at-RA can bind to insulin-like growth factor-II receptor and enhances the primary effect of this receptor (Kang et al., 1999). It has also been found that at-RA can modulate the activity of protein kinase C by direct binding to the retinoic acid binding site of protein kinase C-α (Radominska-Pandya et al., 2000). Finally, retinoylation (acylation by RA of proteins) is another mechanism by which RA may act on cells (Takahashi and Breitman, 1994).

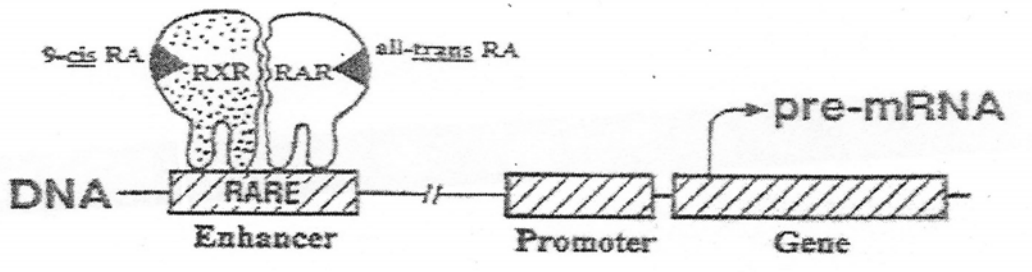


Figure 10. The mechanism behind retinoic acid regulated gene expression. Retionic acid binds to RAR which dimerize with RXR and RAR-RXR heterodimer bind to RARE (retinoic acid response element) (Blomhoff, 1994).

1.4.3 Vitamin A and immunity

It is well established that vitamin A plays an essential role for normal immune function (Blomhoff and Smeland, 1994; Nauss, 1986; Ross, 1992; Semba, 1999; Sommer, 1993a; Stephensen, 2001; Villamor and Fawzi, 2005). This conclusion is based on numerous studies in animals and humans, studying the effects of vitamin A deficiency and supplementation.

Vitamin A deficiency in humans is associated with increased morbidity and mortality from infections, such as for example respiratory-, diarrheal-, and -gastrointestinal infections (Semba, 1999; Sommer, 1993b). Deficiency can be a result of too low dietary intake of the vitamin and /or from severe or chronic infectious diseases, which causes depletion of vitamin A through several mechanisms (Stephensen, 2001). Clinical trials suggest that vitamin A-supplementation reduces morbidity and mortality from different infectious diseases, such as measles, diarrhoeal disease, measles-related pneumonia and human immunodeficiency virus infections (Semba, 1999; Semba et al., 2005). However, how vitamin A improves immune function against these infections is poorly understood (Semba, 1999). Vitamin A as an immunostimulating agent is further proven by studies where vitamin A supplementation increased the efficiency of several vaccines, such as diphtheria, measles and polio type 1 (Bahl et al., 2002; Benn et al., 2002; Ross, 1992). In addition, vitamin A, either in the form of retinol or its metabolite, retinoic acid, has been shown to stimulate the rejection of certain immunogenic tumors (Ross, 1992).

Most of our knowledge on how vitamin A influence the immune function come from studies where the immune system of vitamin A-deficient (VAD) or normal animals has been challenged (see review from (Blomhoff and Smeland, 1994; Nauss, 1986; Ross and Stephensen, 1996; Stephensen, 2001). From these studies it is obvious that vitamin A can affect components of all parts of the immune system. For instance, vitamin A can fortify the innate immune system by stimulating natural killer cells and macrophages, and in

addition vitamin A is involved in the regeneration of mucosal barriers (Blomhoff and Smeland, 1994; Ross and Stephensen, 1996; Stephensen, 2001). Also, both humoral and cellular immune responses can be stimulated by vitamin A (Blomhoff and Smeland, 1994; Stephensen, 2001)).

1.4.4 Effect of vitamin A on isolated T lymphocytes

Previous studies on the role of vitamin A in T cells have been conflicting, since they have indicated that vitamin A could enhance, inhibit, or have no effect on T cell proliferation (Blomhoff and Smeland, 1994; Fish et al., 1981; Nauss, 1986). However, these conflicting data could in part be explained by differences in concentration of vitamin A and the purity of the T cell (Blomhoff and Smeland, 1994; Fish et al., 1981; Nauss, 1986). It was found that while concentrations of atRA above 10 μM was inhibiting, lower concentrations (in this study 0.1-10 μM) enhanced PHA- induced proliferation of bovine lymph node cells (Mastro and Pepin, 1982). In another study it was found that while atRA (at 50nM-5 μM concentrations) enhanced both TPA- and PHA-induced proliferation of highly purified human peripheral blood mononuclear cells, RA had various and inconsistent effects on unpurified peripheral blood mononuclear cells (PBMCs) (Valone and Payan, 1985). Various in vitro studies in the 1980`s and 1990`s using different T cell-activating stimuli indicated that RA or retinol can stimulate the proliferation of for example: mouse spleen cells (Jiang et al., 1992), antigen-specific mouse T cell clones (Friedman et al., 1993), human thymocytes (Sidell and Ramsdell, 1988)and human PBMCs (Allende et al., 1997). RA did not induce T cell proliferation by themselves in any of those studies, indicating that RA has a co-stimulatory role in T cell proliferation.

Recently, we have demonstrated that purified $CD4^+$ human T cells are stimulated by RA (Ertesvag et al., 2002), and that apoptosis is prevented (Engedal et al., 2004).

Except from studies in our own group, the molecular mechanism(s) on how vitamin A might stimulate T cell proliferation has been poorly studied. We observed that the effect

of RA on both T cell proliferation and on apoptosis, at least in part was dependent on the ability of RA to increase IL-2 production in human T cells (Engedal et al., 2004; Ertesvag et al., 2002). It has also been reported that RA increase the expression of IL-2 receptors in murine splenic T lymphocytes (Jiang et al., 1993) and in human thymocytes (Sidell et al., 1993). Furthermore, Sidell and coworkers have studied the effect of retinoic acid on the expression of IL-2Rα- and-β in Hut78 cells (a mature T cell-line) and found that RA can upregulate IL-2R and increase the activation state of these cells (Sidell et al., 1997).

2. AIMS OF THIS THESIS

Vitamin A has been shown to be important for an optimal function of the immune system. Numerous studies in man and in animal models have revealed that vitamin A deficiency leads to increased risk of infections, and that supplementation of vitamin A to such individuals restores the immune function (Blomhoff and Smeland, 1994; Ross, 1992; Semba, 1999; Sommer, 1993a; Stephensen, 2001; Villamor and Fawzi, 2005). It is not, however, clear how vitamin A stimulates the immune system. In our lab we have for several years studied the role of vitamin A on purified populations of B-and T-lymphocytes *in vitro*. We have found that proliferation of both human and murine B-cell precursors is inhibited by physiological levels of the vitamin A metabolite retinoic acid (RA) (Blomhoff et al, 1992; Fahlman et al; 1995; Naderi and Blomhoff). Also naive human B-cells are inhibited by RA (Fahlman et al, 1995), whereas the apoptosis in these cells is prevented (Lomo et al., 1998). In contrast to the effects on B-cells, we have shown that the proliferation of normal human T-lymphocytes is stimulated by RA (Ertesvag et al., 2002), and that also in T-cells, the apoptosis is prevented (Engedal et al., 2004). Furthermore, we have recently demonstrated that the effects of RA on both proliferation and apoptosis in T-cells are mediated via induced production of IL-2 (Engedal et al., 2004).

The overall purpose of the present study has been to elucidate if RA, independent of its ability to induce IL-2, also is able to enhance IL-2 mediated signaling in T-cells. We have had the following specific aims:

1. Determine whether RA enhances IL-2 mediated proliferation

If so,

2. Determine whether RA affects IL-2 mediated changes in the cell cycle machinery.
3. Understand the mechanisms whereby RA may stimulate IL-2 mediated cell signaling.
4. Determine whether the effects of RA on IL-2 mediated signaling involves the nuclear RARs and/or RXRs.

3. MATERIALS AND METHODS

3.1 Materials

Chemical	Producer
α-^{32}P dCTP (10mCi/ml)	Amersham
Acetic acid	Merck
Acrylamid/Bis	Sigma
Agarose	Gibco/BRL
AG-490	Calbiochem-Novabiochem
Am580	Sigma
Aprotinin	Sigma
Bovine Serum Albumin	Sigma
β-mercaptoethanol	Sigma
Bio-Rad Protein assay	Bio-Rad
Bromophenol blue	Bio Rad
Carbonyl iron	Sigma
DMSO	Sigma
EDTA	Prolabo
Ethanol	Arcus
Ethidium bromide (EtBr)	Sigma
Fetal Bovine Serum	Gibco/ BRL
Formaldehyd	Merck
Formamide	Merck
γ-globulin	Sigma
Glutamine	Gibco/ BRL
Glycerol	Prolabo
Glycine	Sigma
HCl	Merck
HEPES	Sigma
rIL-2	R&D systems
Isopropanol	Arcus
Leupeptin	Sigma
LY294002	Sigma
Lymphoprep™	Fresenius Kabi Norge
Methanol	BDH
Microscint™	PACKARD
Molecular weight standard	Bio Rad
MOPS MULTI-CORE™Buffer (10X)	Promega
Non fat dried milk	Nestle Molico
NP-40	Sigma
OPti-fluor®	PACKARD
Penicillin Streptomycin	Gibco/ BRL
Pepsin	Sigma
PMSF	Sigma
Ponceu S	Sigma

Precision Protein Standard Broad Range	Bio-Rad
Propidium Iodide	Sigma
Ro-41-5253	Gift from Dr M Klaus (Hoffman-La Roche, Basel, Switzerland)
RPMI 1640	Gibco/ BRL
Sodium acetate	Prolabo
Sodium azid (NaN_3)	Merck
Sodium chloride (NaCl)	BDH laboratories
Sodium dodecyl sulphate (SDS)	Sigma
Sodium fluoride (NaF)	Merck
Sodium hydroxide	Merck
Sodium orthovandate (Na_3VO_4)	Sigma
Sodium pyruvate (Na-P)	Sigma
Sodium tetra borate	Merck
Retionic acid	Sigma
RNasin	Promega
TEMED	Bio-Rad
[^{3}H]-thymidine	Amersham Biosciences
Tris base	Angus Buffers & Biochemicals
Triton X-100	Sigma
TTNPB	Sigma
Tween20	Sigma
β -glycerophosphate	Sigma
β-Mercaptoethanol	Sigma

Anti bodies

Anti-CD3	Purified from OKT-3-producing hybridoma cell-culture supernatants
Anti CD4 beads	Miltenyi Biotec
Cyclin A (C-19) rabbit polyclonal IgG	Santa Cruz Biotechnology
Cyclin D3 ((DSC-22)	MBL
Cyclin D2 (C-17) rabbit polyclonal IgG	Santa Cruz Biotechnology
Cyclin E (HE 12) mouse monoclonal IgG	Santa Cruz Biotechnology
FITC-conjugated anti-BrdU	PharMingen
Goat anti –mouse IgG	Bio-Rad
Goat anti-rabbit IgG	Bio-Rad
p21 (C-19)rabbit polyclonal IgG	Santa Cruz Biotechnology
p27 (C-19) goat polyclonal IgG	Santa Cruz Biotechnology
Anti phospho-STAT3 (Tyr705, #9135)	Cell signalling Technology
pRB (14001A) Mouse monoclonal IgG	Pharmingen
Phospho-STAT3 (Ser727, #9134)	Cell signalling Technology
STAT3 (#9132)	Cell signalling Technology

Kits

ECL+Western Blotting detection system	Amersham Pharmacia Biotech
Mega prime DNA labelling system	Amersham

Rneasy Mini Kit	QUIAGEN
Vectors	
Cyclin D3 vector (pEF1-HisC)	Gift from Soheil Naderi
Enzymes	
RNase A	Sigma
Equipment	
Agarose electrophoresis apparatus	Hoefer
Beckman Optima™ LE-80K Ultracentrifuge	Beckman
Buffy coat	Ullevål University Hospital
CastAway Gel Dryer	Stratagene
CastAway® Precast Sequencing Gel	Stratagene
Cell culture flasks	NUNC
Cell culturing plates (24- and 96-well flat-bottomed microtiter plates)	BD; Falcon 3072 and 3047
Centrifuge tube (15ml and 50 ml)	Merck
CO2-incubator	Nuaire™
Coulter® Microdiff 18 (Cell counter)	Dan Meszantsky
Disposable syringe (1 or 2ml)	Becton Dickinson
Eppendorf tubes (micro tubes 1,5 ml)	Axygen Scientific
Erlenmeyer bottles	Pyrex
FaCSCaliber	Becton Dickinson
Falcon tubes (5ml)	Beckton Dickinson
Filtermate 196 harvester	Packard
21G needles	Becton Dickinson
Geiger counter	Laborel
Gladpack™	Andvord
Heating block	Dan Meszantsky
Hybaid hybridization oven	Hybaid
Hybond™ ECL ™ Nitrocellulose membrane	Amersham Pharmacia Biotech
Hybond™-N	Amersham LIFE SCIENCE
Hybridization cylinder	Hybaid
Hyperfilm™ MP	Amersham Pharmacia Biotech
Incubator Shaker	New Brunswick scientific co
Kodak X-Omatic cassette	Kodak
Light microscope equipped with a 40x objective	Nikon
LS 6500, Multi-purpse scintillation counter	Beckman
Magnet for cell culture flask	Self-made
Micro tube (0,2 ml)	Abgene
Micro tube (1,5ml)	Axygen Scientific

Microwave oven	Sharp
Mini protean II gel apparatus	Bio Rad
Packard TopCount™ (harvester and microplate scintillation counter)	Packard
Parafilm M	Kebolab
Platform shaker (S25)	Edmund Blücker
Polyetylene (PE) Vials (6ml) with caps	Packard
Polystyrene Round-Bottom tube (5ml)	Falcon
Rotator mixer	Stuart scientific
Semicro disposable cuvettes (1,5ml)	Plastibrand
Sephadex G-50 NICK® Columns	Pharmacia Biotech
Spectrophotometer, Ultrospec 3000	Pharmacia Biotech
Sterile scissors	Rocket Medical
Syringes (1, 2 or 5ml)	Terumo
TC-Plate, 96 well, U-shape	Greiner
Thin Layer Chromatography plate	Merck
TopCount™	Packard
Trans-Blot® SD Semi-Dry Transfer Cell	Bio Rad
Ultrospec 3100 pro spechtrophotometer	Amersham Biosciences
Unifilter®-96, GF/C®	PerkinElmer
UV transilluminator	Saveen
Vortexer	Heidolph
Water bath	Julabo
Whatman® Chromatography paper 3MM	Whatman

3.2 Isolation and culturing of normal T-lymphocytes

3.2.1 Isolation of PBMC

Peripheral blood mononuclear cells (PBMC) were separated from buffy coats of human blood donors, admitted to the Blood Bank at the Ullevål University Hospital in Oslo. A buffy coat contains lymphocytes, some platelets and monocytes, and is obtained by removing erytrocytes and plasma from full blood of normal donors. PBMC were isolated by use of Ficoll-Hypaque density gradient centrifugation (Boyum, 1968). The separation-medium containing sodium-metrizoat and polysaccharide has a greater density than lymphocytes, so that these lymphocytes will be gathered on top of the reagent after centrifugation. Erythrocytes and granulocytes have an even greater density and will form a pellet in the bottom of the tube (see figure 11). It is inevitable that some platelets are isolated together with the lymphocytes, but the majority of these are removed by repeated washing and centrifugation. The desired T-cells will then be isolated by positive selection using magnetic beads coated with antibodies against CD4 (Miltenyi Biotec, Bergisch Gladbach, Germany), as described by the manufacturer. This procedure gives a great yield of T-lymphocytes, but the disadvantage is the high cost of magnetic beads.

Equipment	**Reagents and solutions**
Tissue culture flasks 250 cm^2	Buffy coat
Sterile scissors	Magnetic CD4 microBeads
Plastic tubes 15 ml	RPMI/PS/Gln
Plastic tubes 50 ml	RPMI/ 0.01M EDTA
Magnet adjusted to tissue culture flasks	Carbonyl iron 70mg/ml
Centrifuge	10% FBS
Incubator	Ethanol 70%
	Lymphoprep

Fetal bovine serum (FBS)

Before FBS is added to the RPMI 1640-medium, the serum has to be heated at 56ºC in at least 30 minutes to inactivate complement. FBS is kept at -20 ºC. The additions of FBS to RPMI-medium are reported in %.

RPMI 1640 with penicillin, streptomycin and extra glutamine:

RPMI 1640 – medium	500 ml
Penicillin 5000 IE/ml-Streptomycin (PS) 5mg/ml	5 ml
Glutamine 2mM	5 ml

The medium is kept in the refrigerator at 4ºC.

Buffer for isolation of CD4+ T cells:

FBS (0.5% (v/v))	500µl
EDTA (stock: 0.5M) 2mM	320µl
PBS (phosphate buffered saline pH 7.2) to	100 ml

Procedure

The isolation of PBMC is carried out aseptically in a workstation with vertical airflow, and use of a lab-coat and gloves is necessary to protect the individual performing the procedure in case the blood is infected.

Six tubes each with 15 ml Lymphoprep are prepared beforehand. The buffy coat tube is sterilized with 70% ethanol, and a sterilized scissors is used to cut the tube off. The buffy coat is pored into a culture flask containing 160 ml cold RPMI/ 0.01 % EDTA. 35 ml of this mixture is carefully layered on top of the Lymphoprep in each of the six tubes. The tubes are centrifuged at 600 x g (with minimum brake) at room temperature for 20 minutes. The red blood cells will now occur at the bottom of the tube, while a white layer of mononuclear cells (MNC: lymphocytes, macrofages and monocytes) appears on top of the Lymphoprep layer.

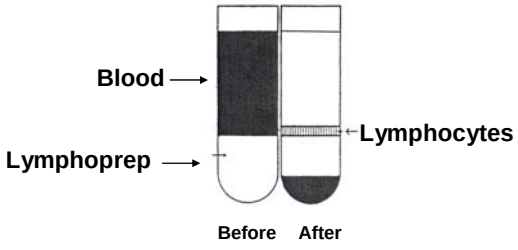


Figure 11. Before and after density gradient sentrifugation.

The mono nuclear cells from the six tubes are gently collected and placed on ice, before centrifugation at 500 x g for 10 minutes. About 3 ml of the supernatant is removed from each tube, and thereafter the tubes are rotated carefully to separate the platelets from the MNC-pellet. The remaining supernatant containing platelets is removed, and after resuspending the MNC-pellet in 7 ml RPMI, the cells are recollected in a 50 ml tube prior to centrifugation at 500 x g for 10 minutes.

The supernatant is removed, and the pellet is resuspended in 10 ml RPMI/10% FBS. 1 ml carbonyl iron with ethanol per 400 million cells is added to a 14 ml tube. The tube is placed on a magnet, the ethanol is removed and the carbonyl iron is washed twice with RPMI (5ml) and once with RPMI/10% FBS (5ml), before the carbonyl iron is resuspended in 5ml RPMI/10% FBS and placed in a culture flask. Cells and RPMI/10%FBS is added to a final cell concentration of 1.0×10^7 cells/ml. After incubating at 37 ºC for 45 minutes, the culture flask is placed on a magnet and the supernatant is transferred to a 50 ml tube on ice. The macrophages have now engulfed the carbonyl particles and are removed. The lymphocytes are counted and collected by centrifugation at 500 x g for 10 minutes.

Isolation of CD4+ T-cells

The isolation of $CD4^+$ T-cells is now performed by using magnetic CD4 beads, and it is done according to the manufacturer's description with only minor changes. It is important to work fast and to keep the solutions cold to avoid capping of antibodies at the cell surface and nonspecific binding of antibodies to the cells.

The cells are washed with 1-2 ml buffer/10^7 cells by centrifugation at 500 x g for 10 minutes. The supernatant is removed and the cell pellet is resuspended in 80 µl buffer/10^7 cells. The magnetic CD4 beads are added to the cells (20 µl of the magnetic beads/10^7 cells) and the cells are placed at 4-8 º C for 15 minutes. After centrifugation at 500 x g for 10 minutes, the supernatant is removed and the cells are resuspended in 500 µl buffer/10^8 cells.

The cells are now transferred to a LS (MACS) column that is placed on a magnet. All the cells that are bound to CD4 beads (CD4+ T cells) will remain in the column. CD4- cells will pass through the column and are removed. The column is washed with 3 ml buffer. The column is removed from the magnet, placed on top of a suitable collecting tube, and thereafter the CD4+ cells are flushed out by using the stamp. The cells are washed in 9 ml RPMI and collected by centrifugation at 500 x g for 10 min. The cell pellet is resuspended in ex vivo medium at a density of $5x10^6$cells/ml.

3.2.2 Culturing normal T-cells

When keeping cells in culture, it is essential to use aseptic technique to avoid contamination of microorganisms. As an extra precaution, the cell culture medium is often supplemented with antibiotics, penicillin and streptomycin (PS), to prevent growth of bacteria. The cells are grown in an incubator at 37ºC, with 5 % CO2 and humidified air to simulate an in vivo situation. When taken out of the incubator the cells must immediately be placed in a sterile cell culture hood. Gloves should be worn, and all equipment used should be sterile or washed with ethanol before brought into the hood.

Normal T cells are grown in RPMI-medium. This medium contains inorganic salts, nutrients as amino acids and vitamins, glucose as an energy source, gluthatione and pH-indicator. Glutathione (glutathion S-transferase) is a phase II biotransformation enzyme, which protects against oxidative damage. Extra glutamine is also added. No serum is added to the culture medium.

Equipment
Microtiter plates with 96- and 24-wells
Incubator
Sterile pipettes

T cells were cultured in standard 96-or 24-well flat bottomed microtiter plates, at a density of 1,5x 10^6 cells/ml in RPMI 1640 supplemented with extra glutamine and PS.

3.3Methods for measuring cell proliferation

3.3.1 Cell counting

The Coulter counter is an electronic cell counter based on the principle that the electrical resistance in an electrolyte solution will change when particles pass the current. As each cell passes through a small aperture it impedes the current and causes a measurable pulse. The number of pulses signals the number of particles. The procedure was performed according to the manual of the Coulter Microdiff-18 instrument, and cells would be counted at a range between $0{,}1x10^6$ to $50x10^6$.

3.3.2 Measure of DNA-synthesis by ^{3}H-thymidine uptake.

Cells that are activated into S phase will usually proceed to mitosis and eventually divide. Thus, incorporation of radiolabeled nucleic acid precursors, such as thymidine, into DNA during S phase of the cell cycle, is a widely used method to measure cell proliferation. The cells are harvested onto a glass fiber filter, free nucleosides removed, and the amount of radiolabeled thymidine incorporated into DNA is measured in a scintillation counter.

Equipment

Microtiter plate with 96 wells
Packard FilterMate cell harvester
Glass fiber filter (Unifilter -96 GF/C filters)
Topcount liquid scintillation counter (Packard)
CO_2-incubator

Reagents and solutions

RPMI/PS/Gln
[^{3}H]-Thymidine 1000 mCi/ml
MicroScint cocktail (Packard)

Procedure

T-cells are placed in 96 well flat-bottomed microtiter plates, in a total volume of 200 µl. The cells are incubated from 1-3 days, depending on what experiments that are being done. To each well is added 50 µl [^{3}H] - thymidine (20mCi) and the cells are incubated for another 16-18 hours. The cells are harvested onto a glass-fiber filter (Unifilter-96 GF/C filters) by using a semiautomatic cell harvester, before registration of incorporated [^{3}H] thymidine in a scintillation counter according to the instrument manual. All samples are grown in triplicates in order to get statistically valuable data.

3.3.3 Determination of cell cycle distribution

The classical method for determining cell cycle distribution is to simultaneously measure incorporation of bromodeoxyuridine (BrdU), and DNA content by flow cytometric analysis. BrdU is incorporated into the DNA of S phase cells and detected by using FITC-conjugated antibodies against BrdU. Anti-BrdU antibodies only recognize BrdU in denatured DNA. Therefore, DNA must be denatured in situ by means of alkali, acid or heat. Increased BrdU-incorporation in S phase cells and a doubling of the DNA content in G2 phase cells enables a separation of cells in G1, S and G2 phases of the cell cycle. The analysis is done by the use of a fluorescence activated cell sorter (FACS). This equipment measures and analyses optical properties of single cells passing through a focused laser beam. When cells pass through the laser beam, they disrupt and scatter the laser light, which is detected as forward scattered (indicates cell size) and side scattered light (indicates granularity of the cells) (Dolbeare et al., 1983).

Equipment

24-well cell culture plates

CO_2-incubator

Plastic tubes 15 ml

Polystyrene round bottom tubes

Flow cytometer (FACSCaliber)

Reagents and solutions

RPMI/PS/Gln (see section 3.1.1)

FITC-labeled BrdU

PBS

70% ethanol

Pepsin-HCl solution

Sodium tetra borate pH 8.5

IFA (Immunofluorescence Assay)-buffer

IFA-Tween

Ribonuclease A/ Propidium iodide solution

Pepcin-HCl:

Pepcin	0.3mg
HCl 2M	to 1000 µl

IFA-buffer:

NaN_3	1ml
FBS	40 ml
HEPES buffer 10X	96ml
Distilled water to	1000 ml

IFA-T:

IFA	199ml
Tween	1ml

Antibody-BrdU-solution:

FITC-conjugated anti Brdu- antibody	10 µl
IFA	112 µl

PI/Ribonuclease A solution:

PI (stock=1mg/ml) 20 µg/ml	80µl
RNase A (stock=10mg/ml) 40 µg/ml	16µl
PBS	to 4 ml

10x HEPES-buffer:

HEPES	95.2g
NaCl	350.8g
Destilled water to	4 l
pH is adjusted to	7.4

Procedure

Cells (300 000-500 000 cells/sample) are incubated for a chosen period of time. BrdU (10µM) is added to cells about 2 hours before harvesting. The cells are then centrifuged at 500 x g for 10 minutes. The pellet is resuspended in 100 µl cold PBS, and 4 ml 70 % ice cold Ethanol is added while vortexing the tubes. The tubes are kept at 20°C for at least 18 hours. Thereafter, the cells are centrifuged at 400 x g for 5 minutes and the pellet resuspended in 1 ml pepsin-HCl for 30 minutes. The acid is neutralized with 3 ml 0.1 M sodium tetraborate (pH 8.5) and the cells are then collected by centrifugation. The cells are washed in 2-3 ml IFA, and after centrifugation the cells are resuspended in 2-3 ml IFA-T and incubated for 5 minutes at room temperature. After centrifugation the pellet is resuspended in 500 µl Rnase/PI solution, and the cells are incubated for 10 minutes in the dark. The cell suspension is transferred to Polystyrene Round-Bottom Tubes through a mesh-film and analyzed in a flow-cytometer according to the manual of the instrument.

3.3.4 CFSE-staining

The overall goal of CFSE-staining cells is to determine the number of cell divisions of a cell population. CFSE (fluorescent-5-(and 6-) carboxyfluorescein diacetate, succinimidyl ester) is a dye that accumulates inside cells and is diluted, when the cells divide. The succinimidyl moiety of CFSE which is highly reactive becomes covalently coupled to nucleophils (e.g. amino acids) forming long-lived fluorescent conjugates that cannot escape from the cell, and this label remains stably associated with the cell. Thus, CFSE-labeled cells can be used to analyze cell division, since the dye is evenly distributed to the progenies resulting in the halving of the fluorescence intensity after each division (Rodel et al., 2005).

Equipment	**Reagents and solutions**
96-well U-bottom cell culture plate	CFSE (10mM)
Polystyrene round bottom tubes	DMSO
Flow-cytometer (FACSCaliber)	PBS
CO_2-incubator	PBS/20% FBS
Plastic tubes 50 ml, 15 ml	RPMI/PS/10%FBS
Centrifuge	RPMI/PS/Gln

Procedure

All handling with CFSE has to be done under the protection from light.

CFSE is dissolved in 90 µl DMSO to a stocksolution at10mM, and the CFSE-solution is kept at -20°C. The cells, about 50-60 millions, are washed once in PBS/0.5% FBS and the pellet is resuspended in 4 ml PBS. The CFSE-solution is diluted 30x in DMSO, and 2 µl of this dilution is transferred to a 50 ml plastic tube. The 4 ml cell suspension is quickly transferred to this tube, and thoroughly resuspended. The cell-CFSE mixture is incubated in a water bath at 37°C and 20 ml PBS/20%FBS is added prior to centrifugation at 500 x g for 10 minutes. This washing step is repeated once prior to incubation for 1-2 hours. After centrifugation, the cell pellet is washed with 14 ml RPMI/PS/Gln/0.5% FBS and centrifuged at 500 x g, before 1 ml of the same medium is added.

3.4 Methods for measuring cell death

3.4.1 Scatterprofile. Measuring the distribution of viable and dead cells

A fluorescence activated cell sorter (FACS) is a powerful method to study different features of cells. When individual cells held in a thin stream of fluid are passed through one (or more) laser beams, they cause light to scatter. The scattered light is detected as forward scattered and side scattered light. Forward scatter approximates cell size, while side scatter is an indicator of the cell`s granularity or complexity (Swat et al., 1991).

Loss of volume and increase of density is a characteristic feature of apoptotic cells. This is a result of apoptotic cells shrinking and being more granular than viable cells. In addition, chromatin condensates, the nuclei fragmentize and apoptotic bodies are formed. Thus, by analyzing the scatter profiles, it is possible to separate cells into viable cells, apoptotic cells and necrotic cells, since necrotic cells will swell, and are therefore larger than viable cells.

3.4.2 PI-staining of cells

By staining cells with Propidium iodide (PI) it is possible to distinguish between dead and viable cells. PI is a dye which binds DNA and is taken up by necrotic and late apoptotic cells, while viable and early apoptotic cells exclude PI, because they have an intact nuclei membrane. PI-staining of the cells is detected as red fluorescence in a flow cytometer equipped by an argon-laser.

Equipment

96 well U-bottom cell culture plate
Polystyrene round bottom tubes
Flowcytometer (FACSCaliber)
CO_2-incubator

Reagents and solutions

RPMI/PS/Gln
Propidium iodide (15µg/ml)

Procedure

All handling of PI should be done under protection from light.

PI (70 µl) transferred to polystyrene round bottom tubes, and 140 µl cell suspension

(0, 2 mill cells) is added. After careful resuspension, the cell mixture is placed in the dark at roomtemperature for 10 minutes, (if desired the cell-suspension can be placed in the refrigerator for 1-2 hours), before it is analyzed for red fluorescence by flow-cytometry (PI-staining). The method is usually combined with analyzing forward and side scatter.

3.5 Western blot analysis

Western blot analysis is a technique used to detect (identify and quantify) specific proteins in a complex mixture of proteins. The protein samples are solubilized with detergents and reducing agents, denatured by boiling and separated electrophoretically on a polyacrylamide gel. The proteins are transferred from the gel to a nitrocellulose membrane and hybridized with antibodies that react specifically with antigenic epitopes displayed by the target protein attached to the membrane. The methods are based on the procedures described by Maniatis, 1989 (Maniatis et al., 1989).

The main steps in Western blot analysis are:

1. Preparation of cell sample for electrophoresis
2. Determination of protein concentration
3. Separation of proteins by electrophoresis on polyacrylamid gels
4. Transfer of proteins from SDS-PAGE gel to nitrocellulose membrane
5. Staining of proteins immobilized on nitrocellulose membranes with Ponceau
6. Blocking of non-specific binding sites for immunoglobulines on the nitrocellulose membrane
7. Incubation of the nitrocellulose membrane in primary antibody directed against the target protein
8. Incubation in horseradish peroxidase conjugated antibody (secondary antibody)
9. Application of detection reagents (ECL solution)
10. Exposure to film

3.5.1 Preparation of cell sample for electrophoresis

The cells are harvested and the proteins are solubilized using an appropriate lyses buffer. To avoid that intracellular proteases digest the target protein, inhibitors of proteases are added in the lyses buffer. It is also necessary to keep the protein extracts on ice at all times to prevent protein degradation.

Equipment

24 wells cell culture plate

Plastic tubes 1,5 ml

Reagents and solutions

Lyses buffer (RIPA-buffer)

PBS

FBS (10%)

RIPA-buffer:

		25 ml
NaF	50mM	2.5 ml (0.5M)
Na_2VO_4	1mM	250 µl
β-glyserophosphate	10mM	0.5 ml (1M)
Tris HCl pH7.4	50mM	1.25ml (1M)
NaCl	150mM	1.5 ml (2,5M)
NP-40	1%	250 µl (100%)
SDS	0.1%	250 µl (10%)
EDTA	0.5 mM	25 µl (0.5M)
PMSF	0.2mM	50 µl (0,1M)
Leupeptin	10 µg/ml	25 µl (10mg/ml)
Aprotinin	0.5%	5 µl to 1 ml
H_2O		18.4ml

Procedure

T-cells are seeded at a concentration of 1.5x 10^6 cells/ml in 24 wells plates. After treating the cells with various activators and/or inhibitors at different time intervals (see the various figure legends), the cells are collected, 10% FBS is added to the cells, and the cells are centrifuged at 500 x g for 10 minutes at 4 º C. The cell pellet is resuspended in 1 ml cold PBS/0.2 % FBS and transferred to 1.5 ml plastic tubes prior to centrifugation at 500 x g for 10 minutes at 4 º C. The supernatant is removed and 45 µl RIPA buffer is added to each tube (5 µl aprotinin and 1 µl PMSF is added to 1 ml RIPA buffer). Each sample is then vortexed every 5 minutes for 30 minutes, before they are centrifuged at 13793 x g for 20 minutes. Whole cells, nuclei and mitochondria - membranes that are not lysated will be collected in the bottom of the tubes. The supernatant is transferred to new 1.5 ml plastic tubes, and the protein concentration is determined, before the samples are quickly frozen in liquid nitrogen and stored at -80 º C.

3.5.2 Determination of protein concentration

The Bio-Rad protein assay is performed according to the method of Bradford (1976).The Bio-Rad Protein assay is a dye-binding assay where a color change occurs from a brownish to an intense blue color when the Coomassie brilliant blue G-250 in an acidic solution binds to protein. The absorbance maximum shifts from 465 nm to 595 nm, and the absorbance at 595 nm is detected by the spectrophotometer

Equipment
Centrifuge
Plastic tubes 1. 5 ml
Spectrophotometer, Ultrospec 3100 pro (Amerskam Biosciences)
Semimicro disposable cuvettes (1.5ml)

Reagents and solutions
γ-globulin 1mg/ml (dissolved in destilled water)
RIPA-buffer
Bio-Rad reagent

Procedure

A Bio- Rad reagent is diluted 1:5 in water, and standards are prepared by adding 1 ml of this solution to each 1.5 ml tube. 2 parallels of standard samples of 5, 10, 15, 20, 25 or 30 µg γ-globuline are prepared. The samples are prepared by adding 2.5 µl lysate to 1 ml of the diluted Bio-Rad reagent. The samples and standards are transferred to disposable cuvettes and the absorbance at 595 nm is measured according to the manual of the spectrophotometer. A standard curve is constructed (based on the mean of the measured standards) to estimate the protein concentration in the samples.

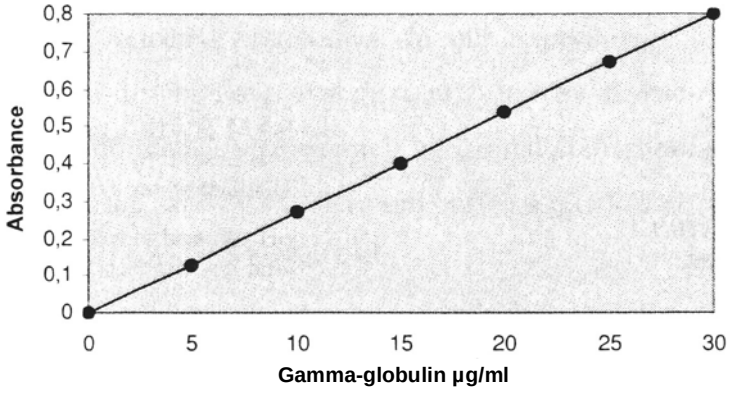


Figure 12. A standard curve showing the correlation between absorbance (595nm) and amount of γ-globulin.

3.5.3 Separation of proteins by acrylamid gel electrophoresis

In gel electrophoresis larger molecules are impeded relative to smaller ones as the molecules migrate through the gel. In polyacrylamid gel electrophoresis, gels are made from polymerization of acrylamid and N, N`-methylenebisacrylamid induced by free radicals. N, N, N`, N`-tetramethylenediamine (TEMED) is added to the gel mixture for stabilizing the free radicals. It is the addition of ammonium persulphate (APS) that generates free radicals. The physical properties of the gel and its pore size are controlled by the proportion of polyacrylamid in the gel and its degree of cross-linking. Typically 12 % acrylamid gels are used for high molecular weight proteins (> 50kDa). The gels have a short "stacking gel"at the top, and a "running gel" below. In the stacking gel the proteins are concentrated into a narrow band, and in the running gel the proteins are separated.

The proteins must be denatured in SDS for 5-10 minutes before loading them on the gel. This is accomplished by boiling the samples in SDS together with the reducing agent β-mercaptoethanol for 5-10 minutes. The denatured polypeptides bind to SDS and become negatively charged.

Equipment and reagents

Bio Rad Mini Protean II gel apparatus
Protein standard, prestained, broad 10%
Acrylamide/Bis
Distilled water
TEMED
Heating block

Solutions/buffers

1.5 M Tris-HCl, pH 8.8:

Tris base	54.45 g
Distilled water	150ml
pH adjusted to 8.8 with HCl	
Distilled water to 300 ml	

3x SDS sample buffer: 10ml

Tris-HCl pH 6.8 1.5M	1166µl
SDS 10%	5140 µl
Glycerol 80%	2260 µl
β-mercaptoethanol	125 µl
Distilled water	1184 µl

Bromophenol blue is added to obtain a visible color.

0,5M Tris-HCl, pH 6.8:

Tris base	12.114 g
Distilled water	to 200 ml
pH adjusted to 6.8 with HCl	

10 x elecrophoresis buffer (pH 8.3):

Tris base	30 g/l	150 g
Glycine	144g/l	720 g
SDS	10 g/l	50 g
Destilled water		to 5 l

10% SDS:

SDS	10 g
Distilled water	to 100 ml

10% APS:

APS	100 mg
Destilled water	to 1 ml

Procedure

The manual from Bio Rad is used when the polyacrylamid gels are formulated and prepared. For identification of $p21^{Cip-1}$, $p27^{Kip}$ and cyclin D we used a 12% separating gel, whereas a 10 % separating gel was used for identification of cyclin A and E. The samples are prepared by dissolving equal amounts of proteins (40-60 µg cell lysate) in 3x SDS sample buffer to a final concentration of 1 x SDS. The samples are boiled for 5-10 minutes, centrifuged at 13000 x g for 1 minute and then loaded onto the gel. The gel is run at 200 V. The separation time depends on the size of the protein which is to be analyzed.

3.5.4 Transfer of proteins from SDS polyacrylamid gel to nitrocellulose

In these experiments a semi-dry transfer apparatus is used for the transfer of the proteins from the gel to the nitrocellulose membrane.

Equipment

Hybond ECL Nitrocellulose membrane
Whatman 3 MM paper
Trans-blot SD Semi-Dry Transfer Cell (Bio-Rad)
Test tube

Solutions

Transfer buffer:

39mM glycine	14.65g
48mM Tris base	29.1g
10 % SDS	18.75ml
Methanol 100%	1000ml
Water to	5 l

The solution is stored at 4ºC.

TBS/Tween (0.1 %) buffer:

Tris base	24.2 g
NaCl	80 g

Tris-base is dissolved in 5 liter destilled water, NaCl is added and then pH is adjusted to 7.6 with concentrated HCl. Tween 20 is added to a final concentration of 0.1%

Procedure

The stacking gel is removed and the running gel is equilibrated in transfer buffer. One piece of nitrocellulose filter and six pieces of Whatman 3MM paper is cut at the exact size of the gel, and placed in transfer buffer. 3 pieces of Whatman paper is placed in the transfer cell and the filter is put on top of the papers. The gel is placed on top of the filter and finally the three last Whatman papers are put on top of the gel. To remove any air bobbles, a glass tube is rolled over the “sandwich”. The transfer is carried out at room temperature at 17 V for 25 minutes.

3.5.5 Staining of proteins immobilized on nitrocellulose membranes with Ponceau S

Staining of proteins with Ponceau S is essential to provide visual proof that transfer of proteins from the gel to the nitrocellulose filter has taken place, and in the same time as an extra control of equal loading. Ponceau S is a rapid reversible protein stain, which is easily washed off during the further processing of the Western blot.

Reagents and solutions

Ponceau S

TBS/Tween (See section 3.5.4)

Ponceau S:

Ponceau S 0.5 g

Acetic acid 1 ml

Distilled water to 100 ml

Ponceau S is dissolved in 1 ml acetic acid. The volume is then adjusted to 100 ml with distilled water.

Procedure

The filter is incubated in a tray containing Ponceau S for about 5 minutes. The filter is then washed several times with distilled water, and eventually with TBS/Tween, in order for the red color to be washed off.

3.5.6 Blocking of non-specific protein binding sites on the nitrocellulose membrane

To reduce the background of nonspecific binding of irrelevant proteins, the nitrocellulose filter is incubated in a solution of nonfat dried milk protein.

Solutions

Blocking solution:

5% Nestle Molico nonfat dried milk in TBS/Tween (0.1%) buffer.

TBS/Tween (0.1% buffer:

See section 3.5.4.

Procedure

A 5 % blocking solution is made by mixing 50 ml of TBS-Tween solution with 2.5 g dried milk and transferred to a tray. The filter is incubated in the blocking solution for one hour under gentle agitation.

3.5.7 Incubation of the nitrocellulose membrane with antibodies

The blot is incubated with an unlabeled antibody (the primary antibody) specific to the protein of interest, in the presence of blocking solution. The blot is then washed, before it is incubated with the secondary antibody which is coupled to an enzyme, horseradish peroxidase. After further washing, the antigen-antibody complex is visualized by chemiluminescence and autoradiography.

Reagents and solutions

Rabbit polyclonal IgG against cyclin D3, cyclin D2, cyclin A, $p21^{Cip-1}$ or $p27^{Kip1}$ or mouse monoclonal IgG against human pRB or cyclin E.

Nonfat dried milk

Sodium azide (NaN_3)

TBS/Tween

Goat anti-mouse IgG or goat anti rabbit IgG (for the mouse monoclonal or rabbit polyclonal IgGs respectively).

Primary antibody solution for cyclin D3, cyclin D2, cyclin A, $p21^{Cip-1}$ or $p27^{Kip1}$ and pRB:

Primary antibody against cyclin D3, Cyclin D2, cyclin A, $p21^{Cip-1}$, $p27^{Kip1}$ or pRB	1µg/ml
Nonfat dried milk	0.5 % (final conc.)
Sodium azide	0.1% (final conc.)

TBS/Tween (0.1%) buffer to 7 ml.

Secondary antibody solution for cyclin D_3 , cyclin D_2, cyclin A, cyclin E, $p21^{Cip-1}$, $p27^{Kip1}$ and pRB:

Peroxidase labeled goat anti-rabbit(against cyclin D_3 , cyclin D_2, cyclin A, $p21^{Cip-1}$ or $p27^{Kip1}$) or goat anti-mouse IgG (against cyclin E or pRB).	1:6000
Nonfat dried milk	2% (final c.)

TBS/Tween (0.1%) to 5 ml

Procedure

The blot is incubated with the primary antibody solution for about 2 hours at room temperature or over night at 4º C under careful agitation. The blot is washed for 10-15 minutes four times in TBS/Tween, before the blot is incubated with the secondary antibody solution for 1 hour at room temperature under gentle agitation. Finally, the blot is washed five times with TBS/Tween.

3.5.8 Detection of proteins by chemiluminescence and autoradiography

The ECL Plus Western blotting detection system produced by Amersham Pharmacia Biotech provides a method for the detection of immobilized antigens conjugated to horseradish peroxidase (HRP) labeled antibodies. The combination of HRP and peroxide catalyzes the oxidation of the Lumigen PS-3 acridian substrate which generates thousands of acridinum ester intermediates per minute. These intermediates react with peroxide under slight alkaline conditions to produce a sustained, high chemiluminescence with maximum emission at a wavelength of 430 nm.

Equipment

ECL Plus™ Western blotting detection system
Hyperfilm MP
Film cassette
Glad pack

ECL Plus™ Western blotting system:

Reagent A
Reagent B

Procedure

Detection of proteins by chemiluminescence is performed according to the manufacturer's description. Reagents A and B are mixed in a ratio of 40:1. The blot is placed on a piece of Glad Pack and excess TBS/Tween is drained off. The mixture of A and B is pipetted on to the membrane and the membrane is incubated for 5 minutes at room temperature. Excess detection solution is drained off, and the membrane is laid in a plastic folder with the protein side up, and put in a film cassette. A sheet of Hyperfilm

MP is placed on top of the membrane in a dark room; the film is exposed for 5 seconds to 5 minutes, and then developed.

3.6 Northern analysis

3.6.1 Isolation of total-RNA from T-cells

RNA is easily degraded by intracellular Ribonucleases (RNase). When working with RNA, it is therefore extremely important to avoid contamination of RNases from any sources. Various precautions must be followed in order to create and maintain an RNase-free environment. Good aseptic technique should be used when working with RNA, and the isolation procedure should be done as fast as possible. Gloves must be changed frequently, and tubes are kept closed whenever possible. Isolated RNA must be kept on ice.

Equipment and reagents

RNeasy Mini Kit
Plastic tubes 1.5 ml
20-gauge needles
Syringes (1-2 ml)
Centrifuge
Rnasin

Solutions

PBS/0.5% FBS
Ethanol

RNeasy Mini Kit includes:

β-Mercaptoethanol
Buffer RLT
Buffer RW1
Buffer RPE
RNase-free water
RNeasy columns
2 ml and 1.5 ml collection tubes

RLT-buffer

β- Mercaptoethanol	10 µl
Rnasin	5.2µl
RLT-buffer	to 1 ml

Procedure

Total-RNA was isolated by the use of Qiagen RNeasy Mini kit. This technology takes the advantage of the selective binding properties of a silica-gel-based membrane, and combines it with the speed of microspin technology. Up to 100 µg of RNA longer than 200 bases can bind to the RNeasy silica-gel membrane because of a specialized high-salt buffer system. First, biological samples are lysed and homogenized by a highly denaturing guanidine isothiocyanate (GITC)-containing buffer, which immediately inactivates RNases to insure isolation of intact RNA. To get appropriate binding conditions, ethanol is added. Thereafter the sample is applied to an RNeasy column where the total RNA binds to the membrane, and contaminants are washed away. Finally, RNA is eluted in 30µl, or more, of water.

The maximum amount of starting material is 1.0×10^7, and the isolation of total-RNA was performed according to the manufacturer's manual:
The cells (10×10^6) are subjected to centrifugation at 500 x g for 5 minutes. 1 ml PBS/0.5% is added to the pellet, and the resuspended the cell-suspension is transferred to plastic tubes (1.5 ml). After centrifugation at 300 x g for 5 minutes, the supernatant is removed and the cells are resuspended in 600 µl RLT-buffer and homogenized by passing the lysate about 20 times through a 20-gauge needle fitted to a syringe. The RLT-buffer is a highly denaturing guanidine isothiocyanate (GITC)-containing buffer, which immediately inactivates RNases to insure isolation of intact RNA. One volume (350 µl) of 70% ethanol is added and mixed well by pipetting. Ethanol is added in order to get appropriate binding conditions. 700 µl of the sample is applied to an RNeasy column, where the total RNA binds to the membrane. The tube is closed, and centrifuged at ≥8000g for 15 seconds. The flow through is discarded. 700 µl Buffer RW1 is applied to the column. The tube is closed, and after new centrifugation, the flow through is again discarded. The RNeasy column is transferred to a new 2 ml collection tube and 500 µl Buffer RPE is added. The tube is closed and again subjected to centrifugation. The flow through is discarded, another 500 µl Buffer RPE is added, but this time the tube is centrifuged for 2 minutes, to dry the silica-gel membrane. To elute, the column is transferred to a new 1.5 ml collection tube, and 30-35 µl RNase-free water is applied

directly onto the RNeasy silica-gel membrane. The tube is closed, and subjected to centrifugation for 1 minute at ≥ 8000 x g. The column is discarded, and the isolated RNA is stored at -80°C.

3.6.2 Electrophoresis of RNA in agarose gels

RNA is fractionated by electrophoresis through a 1% agarose gel containing formaldehyde. Formaldehyd is a denaturant which prevents intrastrand base pairing, and makes the migration rate of a single-stranded RNA entirely dependent on its length. All handling with formaldehyde should be done in a chemical ventilation hood, due to its great toxicity. The RNA-bands are detected by incubation in a solution of ethidium bromide (EtBr). EtBr slips in between the stacked base pairs of RNA and makes RNA fluorescence under UV-light. After staining with ethidium bromide, only two bands are visible, the 28S- and 18S rRNA. The rRNA bands can be used as an extra control of equal loading.

Equipment

Agarose gel apparatus
Microwave oven
UV transilluminator
Water bath

Reagents

Total-RNA samples
DEPC-treated water
MOPS

10x Running buffer with MOPS (10xRB/MOPS):

0.5M EDTA	10ml
1.5M Sodium acetate	16.66ml
Sodium hydroxide	2g

DEPC-treated water to 490 ml. The solution is then autoclaved. 20.93 g MOPS (3-[N-morpholino] propanesulfonic acid) is added and the pH adjusted to 7.0. The solution is stored dark at 4°C.

Bromphenol blue solution:

Glycerol 70% 1.45ml
10xRB/MOPS 200µl
DEPC-treated water 350 µl
0.03% w/v bromphenol blue

Agarose (1%) with formaldehyde:

Agarose 0.8 g
10xRB/MOPS 8 ml
Formaldehyd 37% 14.4 ml

DEPC-treated water to 80 ml.

3xsample buffer with ethidium bromide:

Formamide (deionized) 800 µl
10xRB/MOPS 160 µl
Formaldehyd 37% 256 µl

1 µl ethidium bromide (10mg/ml) is added before use.

Procedure

The procedure is based on the method described by Maniatis et al (1989).
Agarose (1%) gel with formaldehyde is prepared as follows: Agarose is added to the DEPC-treated water, and the mixture is boiled in a microwave oven until the agarose dissolves. The solution is cooled to about 55ºC, and prewarmed 10x RB/MOPS and formaldehyde (55ºC) are thereafter mixed with the agarose solution. The gel solution is poured into the mold and the comb is set into place. After about 30 minutes at 4ºC, the gel is completely set. The gel is then mounted in the electrophoresis tank, and the comb is carefully removed. Enough volume of 10x RB/MOPS to cover the surface of the gel is thereafter added. The total RNA samples are prepared by adding 2.5 volumes of 3x sample buffer containing ethidium bromide. The solution is incubated in a 65ºC water bath for 10 minutes, before it is placed on ice for 5 minutes. After adding 10 µl cold bromphenol blue solution, the sample is loaded into the slots of the gel. The gel is run at 95V for the appropriate time and then placed on the UV-transilluminator for visualization of the RNA bands.

3.6.3 Northern blotting

Northern blotting ensures that the RNA in the gel is transferred on to a nylon membrane by upward capillary transfer. When the transfer is carried out at a neutral pH, the RNA does not become covalently fixed to the nylon membrane. To form covalently bonds between the RNA and the nylon membrane, the membrane is baked at 80ºC (or exposed to UV irridation at 254 nm) before hybridization.

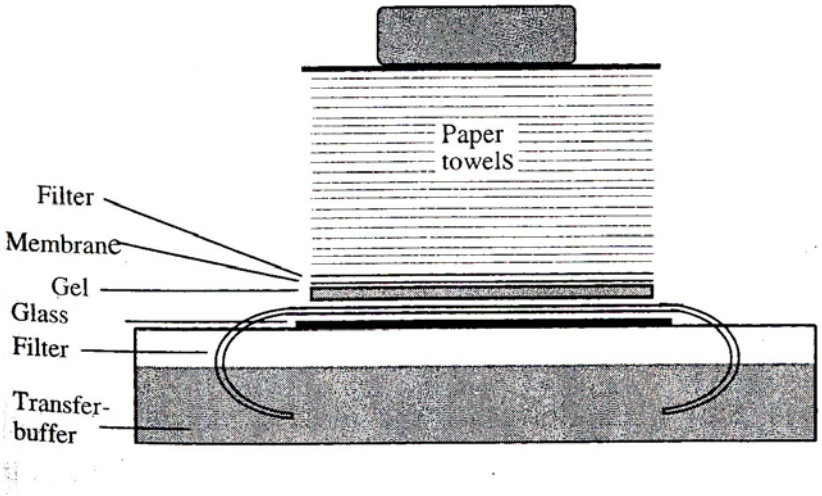
Equipment	**Reagents**
Whatman 3MM paper	DEPC treated water
Parafilm M	
Hybond^{TM}-N	
Glass plates	20xSSC:
Test tube	Sodium chloride 175.3g
Paper towels	Sodium citrate 88.2g
Container	DEPC-treated water to 800 ml
Weight	
Vacuum oven	pH is adjusted to 7.0 with 10M NaOH.
UV-transilluminator	DEPC-treated water to 1000ml.

Procedure

The method is a slightly modified version of the procedure described by Maniatis et al (1989). A glass plate is placed on top of a container containing 20xSSC. 3 pieces of Whatman filter (wider and much longer than the gel) are cut and soaked in 20xSSC, before they are placed on top of the glass plate. It is of great importance that the ends of the filters are well covered with buffer. A test tube is rolled over the surface of the filter paper to remove all air pockets. The gel is then placed on top of the filters in an inverted position. One piece of nylon membrane (1mm larger than the gel in both dimensions) is soaked in DEPC-treated water for 2 minutes and then in 20xSSC for 5 minutes before it is placed on top of the gel. All air pockets between the gel and the membrane are excluded by carefully rolling a test tube over the surface. A stack of paper towels (10 cm

high) is then placed on top of the filter. Finally, a glass plate is placed on top of the stack, and the stack weighed down with a weight (about 500 g). The transfer is run for 16-18 hours. The RNA is covalently bound to the membrane by irridation at 254 nm for 1 minute (or baked at 80 ºC).

The 28 S and 18S RNA bands, which are visualized under UV light, are marked on the membrane by a pencil, and the membrane is baked between two pieces of Whatman filter at 80ºC for 30 minutes. After soaking the membrane in 2xSSC, it can be used for pre-hybridization.



Figur 13. Schematic illustration of the Northern blot transfer setup.

The following part of the Northern blot analysis (labeling of probes, hybridization and autoradiography) was done by another person in the lab, since that meant working with radioactivity in a hot lab. I have not participated in a radioactivity course and could therefore not perform these experiments myself.

3.6.4 Labeling a DNA probe by random primer method

The Klenow fragment of E. coli DNA polymerase I is used to label probes by the random priming method. DNA fragment identical to the RNA of interest is used as a template. Random-sequence primers, 6-9 bases in length, are annealed to the DNA fragments and

incubated with the Klenow fragment and radiolabeled dNTPs. The primers are extended to generate double stranded DNA. To separate the radiolabeled probe from unincorporated dNTPs, the labeling reaction mixture is run through a Sephadex G-50 NICK® column. The amount of incorporated radioactivity is then measured by a scintillation counter.

Equipment and reagents

DNA template
Water bath
Heating block
α-^{32}P dCTP (10mCi/ml)
Sterile water
0.5M EDTA
Mega primer DNA labeling system
Sephadex G-50 NICK® Columns
Microcentrifuge tubes
Opti-fluor®
Polyethylene (PE) Vials (6ml) with caps
LS 6500, Multi-purpose scintillation counter

The Mega prime DNA labeling system includes:

dATP, dCTP, dTTP and dGTP
Reaction buffer
Primer
Klenow-Enzyme
Standard DNA
Carrier DNA

1xTE, pH7.5:

1M Tris-HCl, pH7.4	1 ml (final c.10mM)
0.5M EDTA, pH8.0	0.2ml (final c. 1mM)
In sterile water to	100ml

Procedure

30 ng DNA template, 5 µl primer solution and sterile water to a final volume of 26 µl are mixed. The mixture is placed on a heat block at 100ºC for 5 minutes (to denaturize the DNA) before centrifugation at 10000 x g for a few seconds. The following reagents are

thereafter added in the following order: 4µl dATP, 4µl dGTP, 4µldTTP, 5µl reaction buffer, 5µl α-^{32}P dCTP and 2 µl Klenow-enzyme. The reaction mixture is mixed and then incubated in a 37ºC water bath for 15 minutes. The reaction is terminated by the addition of 2 µl 0.5M EDTA before the solution is placed on ice. To separate the radiolabeled probe from unincorporated dNTPs, the labeling reaction mixture is run through a Sephadex G-50 NICK® column. After equilibrating the column with 3 ml 1xTE, pH 7.5, the radio labeled probe is carefully applicated into the column. The solution is absorbed into the gel bed, and then 2 ml 1xTE, pH 7.5 is added to elute the purified sample. Microcentrifuge tubes are placed under the column for sample collection (3 drops in each microcentrifuge tube). 2 µl of each fraction is transferred to 3 ml Opti-fluor®, and the amount of incorporated radioactivity is then measured according to the manual of the LS 6500 Multi-purpose scintillation counter. The two fractions of the highest radioactivity (> 250000 cpm to be regarded as a good probe) are then used in the following hybridization reaction.

3.6.5 Hybridization and autoradiography

To locate the RNA of interest, the RNA samples that have been transferred and fixed to a nylon membrane can be hybridized with a specific radiolabeled probe. After treating the membrane with blocking agents to suppress nonspecific absorption of the probe, the membrane is incubated under conditions that favor hybridization of the radiolabeled probe to the target RNA. The membrane is washed extensively (to remove nonspecific bound probe) before the DNA-RNA complex is visualized by autoradiography.

Equipment and reagents and solutions

Radiolabeled DNA probe

10% SDS

Hybridization cylinder

Nylon gauze

Hybaid^{TM} Hybridization oven

Heating block

Plastic box

Platform shaker (S25)

Geiger counter

Plastic bag

50x Denhardt's solution:

Ficoll	5g
Polyvinylpyrrolidone	5g
BSA	5g

Sterile distilled water to 500 ml.

20xSSC:

See section 3.6.3

Hybridization solution:

20x Denhardt's solution	4ml (final concentration: 5X)
20xSSC	10ml (final conc. 5XSSC)
1M Na-P, pH 6.5	2ml (final conc. 50mM)
10%SDS	0.4ml (final conc.0,1%)
100% Formamide	20ml (final conc. 50%)
5mg/ml Salmon DNA	2ml (final conc. 250µg/ml
In DEPC treated water to	40 ml

40 ml is enough for hybridizing one filter.

Procedure

Two sheets of RNase free nylon gauze and the Northern blot are soaked in 2xSSC. The blot (sandwiched between nylon gauze) is thereafter placed in the hybridization cylinder

with the RNA side facing the inside of the tube. 20 ml of hybridization solution is boiled for 10 minutes and then cooled on ice. The hybridization solution is transferred to the hybridization cylinder, and the blot is prehybridized at 42ºC for 2 hours in a hybridization oven containing a rotating wheel. Another 20 ml of hybridization solution is boiled for 10 minutes before cooled on ice. The radio labeled DNA probe is denaturized by placing the tube on a heating block at 100ºC for 5 minutes. After cooling down on ice, the probe is transferred to the newly prepared hybridization solution. The prehybridization solution is discarded before transferring the hybridization solution containing the radio labeled DNA probe into the hybridization cylinder. The blot is incubated over night at 42ºC in the hybridization oven. After hybridization, the blot is transferred to a plastic box on a platform shaker, and then washed 30 minutes with 2xSSC/0.1%SDS followed by 30 minutes with 1xSSC/0.1%SDS (at room temperature). Further washing is necessary if the background, measured by a Geiger counter, is too high. The blot is carefully dried before sealed in a plastic bag, and the amount of target RNA in the samples is visualized by autoradiography.

4. RESULTS

4.1 The effect of RA on T cell proliferation and its role in stimulating the cell cycle machinery

4.1.1 RA enhances T cell proliferation induced by anti-CD3 antibodies and IL-2

We wished to determine whether RA is able to modulate IL-2-induced mitogenic signaling in T-lymphocytes. Earlier studies in our lab group had reported that RA enhances TCR/CD3-initiated signaling by increasing IL-2 production, and thus stimulates the proliferation of isolated human T cells. In order to evade the contribution to T cell proliferation which was caused by RA-induced IL-2 production, we added IL-2 receptor-saturating concentrations of recombinant IL-2 (4ng/ml) in combination with anti CD3 (2,5µg/ml) antibodies (OKT3). The experiments were performed in the absence of fetal bovine serum (FBS). This was done to avoid retinol which is found in serum, and may be intracellularly metabolized to RA. Thus, retinol could potentially mask the effect of exogenously added RA, and thereby influence the experiments.

As shown in figure 14, RA potently increases T cell proliferation measured by DNA synthesis. We performed a statistical analysis, paired t-test, by using the SPSS program from Windows, to compare the cpm-values of cells from ten different donors stimulated with OKT-3 and IL-2 in the absence or presence of RA. A p-value ($p<0.01$) indicates that the difference between two samples are statistical significant. As indicated in figure 14, the difference between samples treated with OI or OIR were statistical significant.

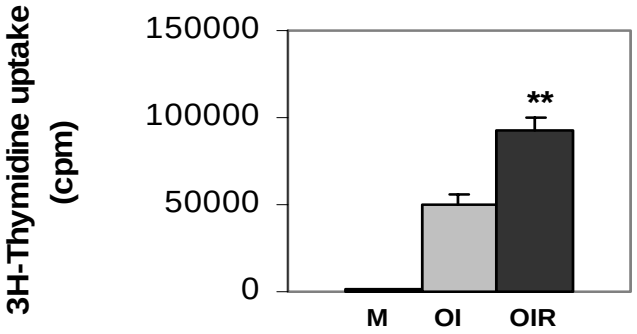


Figure 14. Isolated CD4+ T lymphocytes from 10 different donors were either cultured in medium alone, or stimulated with OKT-3 and IL-2 in the absence or presence of RA (50nM), and after 48 hours they were pulsed with ^{3}H-thymidine for 18 hours, as described in "Materials and methods". Mean cpm-values of ten independent experiments ± SEM are shown (**P<0.01, paired samples t-test). M represents cells cultured in medium alone. OI represent cells treated with OKT-3 and IL-2, and OIR represent cells treated with OKT-3 and IL-2 in the presence of RA.

4.1.2 RA potentiates T cell proliferation in a dose-dependent manner

To investigate whether the effect of RA on T cell proliferation was dose-dependent, we measured the incorporation of ^{3}H-thymidine into DNA after treatment with different concentrations of RA (see figure 15). As illustrated in figure 15, optimal uptake of ^{3}H-thymidine was achieved at 100 nM RA, and effects of RA were observed at concentrations of RA as low as 0.01 nM.

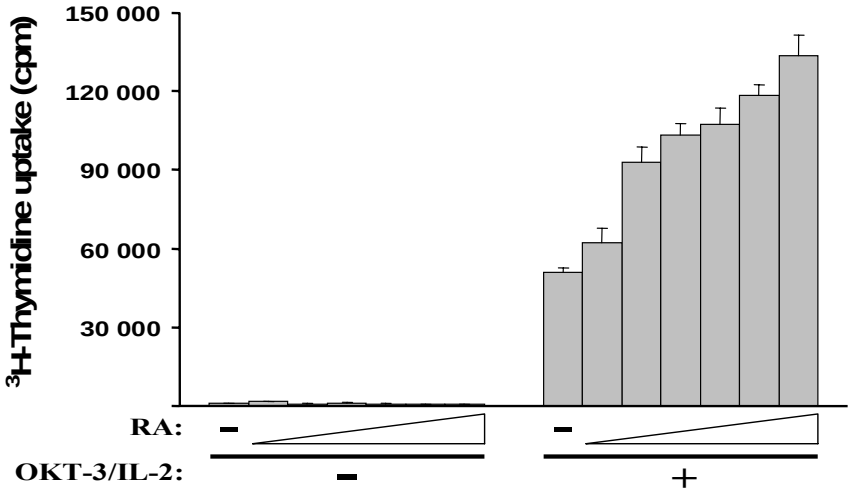


Figure 15. RA potentiates OKT-3/IL-2 induced T cell proliferation in a dose-dependent manner.
CD4+ T lymphocytes were treated with OKT3/IL-2 in the absence or presence of RA at concentrations of RA: 0.01-0.1-1-10-50-100nM. After 48 hours the cells were pulsed with ^{3}H-thymidine for 18 hours, as explained in "Materials and Methods". Mean cpm-values ± standard deviations of triplicates from one representative experiment of three are shown.

4.1.3 RA potentiates DNA synthesis in a time dependent manner

We also wished to determine the kinetics of the RA-effect by measuring DNA-synthesis. As shown in figure 16, the effect was noteworthy at day 2, and RA potently increased DNA synthesis at day 3 and 4. Thus, RA enhances OKT-3/IL-2-mediated T cell-proliferation in a time-dependent manner.

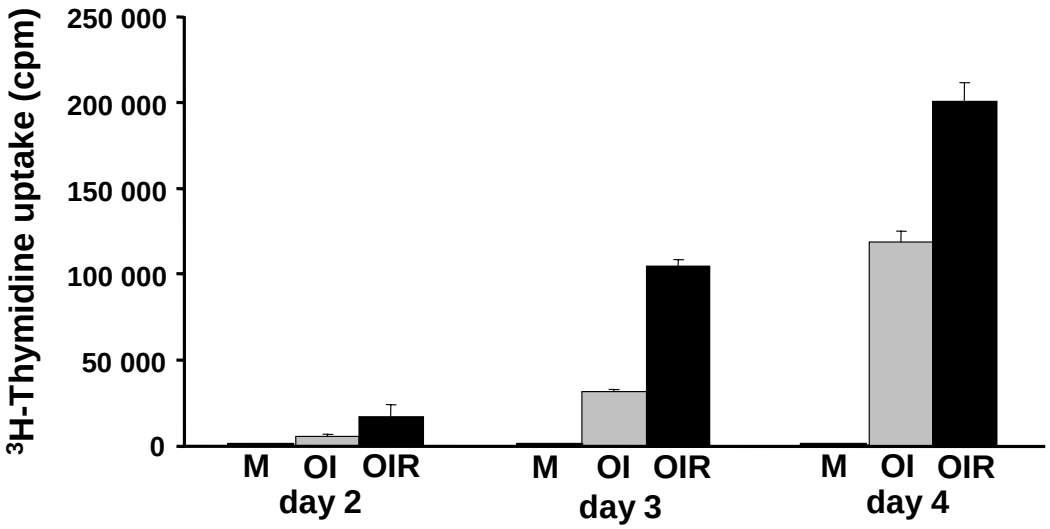


Figure 16. RA potentiates DNA synthesis in a time dependent manner.
Isolated CD4+ T lymphocytes were either cultured in medium alone (M), or stimulated with OKT-3 and IL-2 (OI) in the absence or presence (OIR) of RA (50nM). After 24, 48 and 72 hours, respectively, the cells were pulsed with ^{3}H-thymidine for 18 hours, as described in "Materials and Methods". Mean cpm-values ± standard deviation of triplicates from one representative experiment of three is shown.

4.1.4 RA potentiates T cell proliferation in a time-dependent manner

We next wished to confirm the time-dependent effect of RA that was demonstrated by measuring DNA-synthesis (see section 4.1.3.), by direct measuring cell division. To do so, we stained the cells with CFSE. CFSE is equally distributed between the two daughter cells after cell division, and thus divided cells express half of the fluorescence of undivided cells, and for each new cell division the fluorescence is further reduced. This can be detected by flow cytometric analysis. As illustrated in the dot blot from one representative experiment (figure 17), T cells cultured in medium alone did not undergo cell division. At day 3, however RA had induced one round of cell division in 14% of the T cells (figure 17). At day 4, a larger fraction of cells stimulated with OKT3 and IL-2, had divided once, and a comparable proportion of the cells had undergone two cell divisions in the presence of RA. Hence, 42% of RA-treated T cells had divided, compared to only 10% in the absence of RA. After 5 days, 52% of RA-treated cells had divided, and among these cells a considerable proportion had undergone three cell divisions, whereas only 21% of the cells had divided in the absence of RA. According to

these data it seems that RA stimulates both the initiation and progression of the T cell division process.

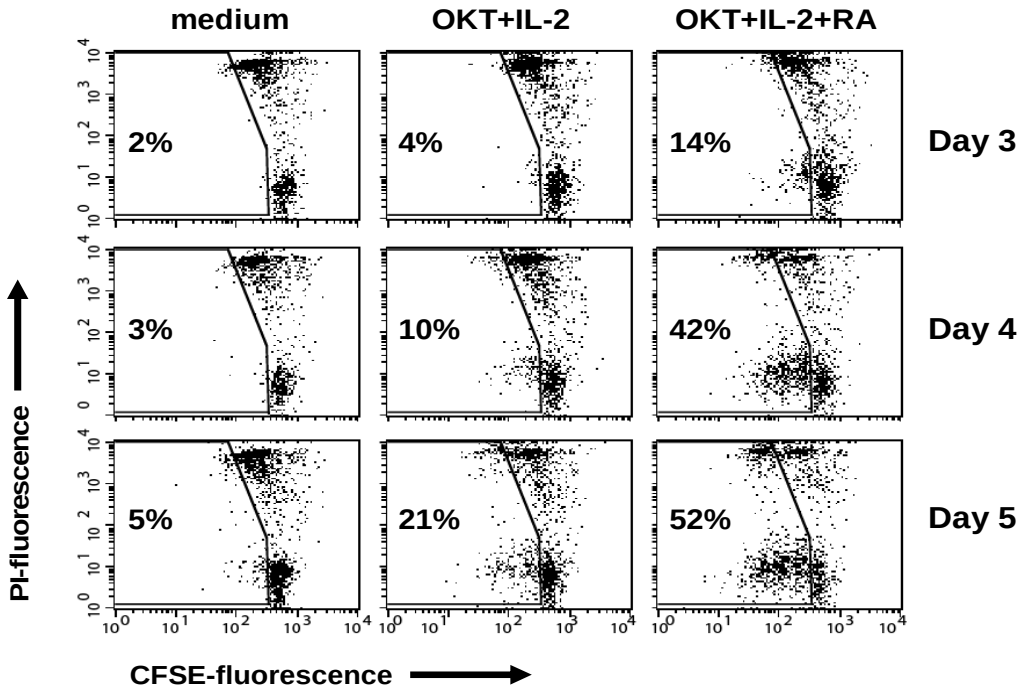


Figure 17. RA potentiates T cell division in a time dependent manner.
T cells were stained with CFSE as described in Materials and Methods, and the cells were treated with OKT-3, IL-2 and RA (100nM) as indicated. The cells were analyzed for percentages of divided cells after three, four or five days, respectively. The gatings were set in order to accommodate for the loss of CFSE-fluorescence by dying cells. Percentages of divided cells are indicated. One representative experiment of eight is shown.

4.1.5 Effect of RA on the cell cycle distribution of T cells

The effect of RA on the cell cycle distribution of isolated T cells was investigated. The distribution of cells in the different phases of the cell cycle was addressed by a combined method of BrdU incorporation, which measures the fraction of cells in S phase, and staining of DNA by propidium iodide, which measures the DNA content of cells. The analysis was performed by the use of flow cytometry. In general, the percentages of the cells that have passed the G1-phase were limited at day 1. At day 3, still most of the cells

cultured in medium alone were in G1, whereas 0.1% of the cells had entered the S-phase (figure 18). In cells stimulated with OKT3 and IL-2, the percentage of cells in S-phase has increased to 3.14%, whereas RA enhanced this percentage of cells to 9.8% (figure 18).

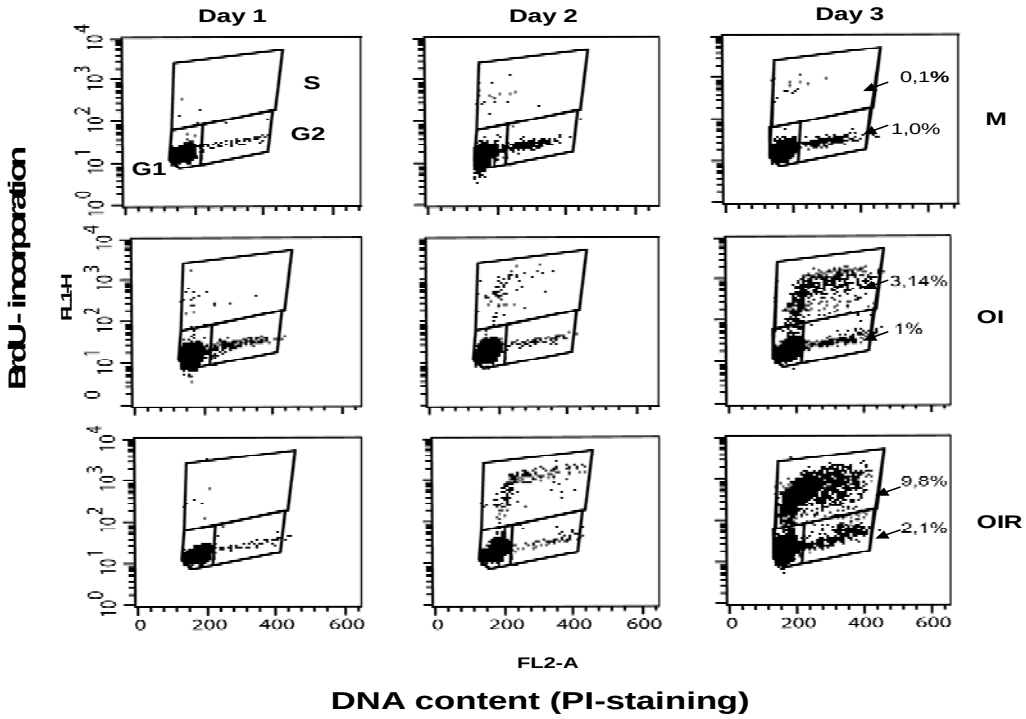


Figure 18. Effect of RA on the cell cycle distribution of T cells.
CD4+-cells were either cultured in medium alone, or stimulated with OKT-3 and IL-2 in the absence or presence of RA (50nM). After 24, 48 and 72 hours BrdU was added at a final concentration of 10µM about two hours prior to harvesting. The cell cycle distribution was determined by flowcytometric analysis after staining the cells with FITC-conjugated antibodies against BrdU in the presence of propidium iodide (PI)/Rnase-solution as described in Materials and Methods. The percentages of cells in S-phase and G2-phase are indicated.

4.1.6 Effect of RA on T cell viability

We next wished to investigate whether RA also is affected by cell death in terms of apoptosis. By flow cytometry it is possible to discriminate between living cells and apoptotic cells. Thus, apoptotic cells are smaller than living cells, and are more granular. Cell size and granularity can be analyzed by forward scatter and side scatter, respectively (figure 19A).

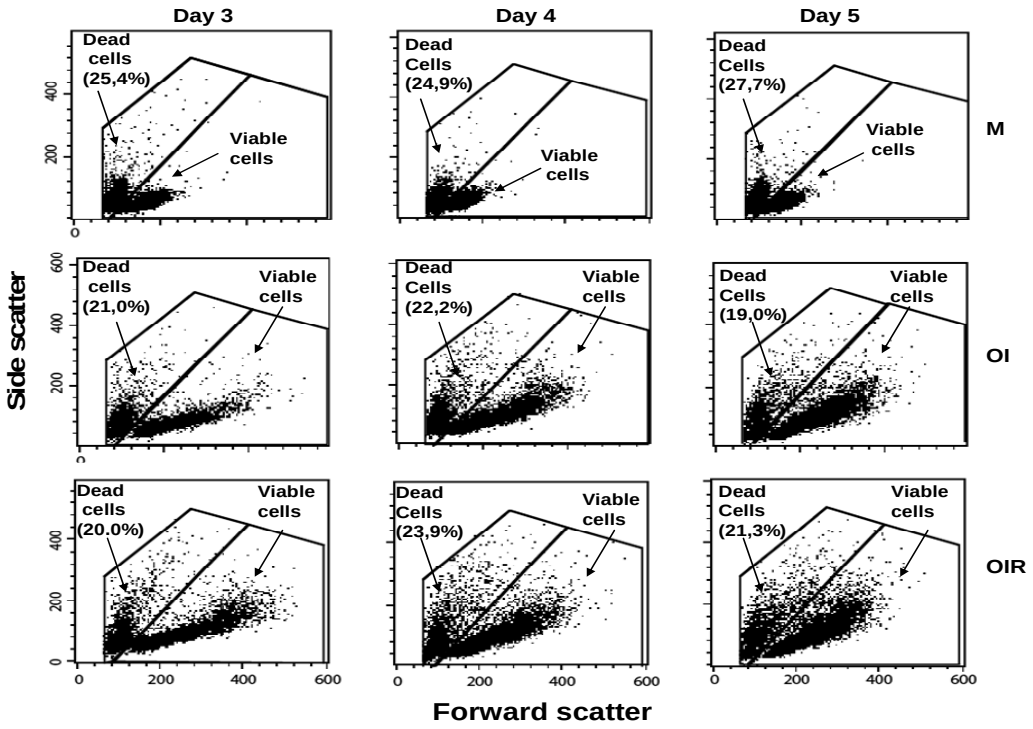


Figure 19A. Effect of RA on T cell viability.
CD4+-cells were either cultured in medium alone (M), or stimulated with OKT-3 and IL-2 (OI) in the absence or presence of RA (50nM). After 3, 4 and 5 days the percentage of dead cells was determined by PI-staining as described in "Materials and Methods". Forward- and side-scatter profiles are illustrated. One experiment of two is shown.

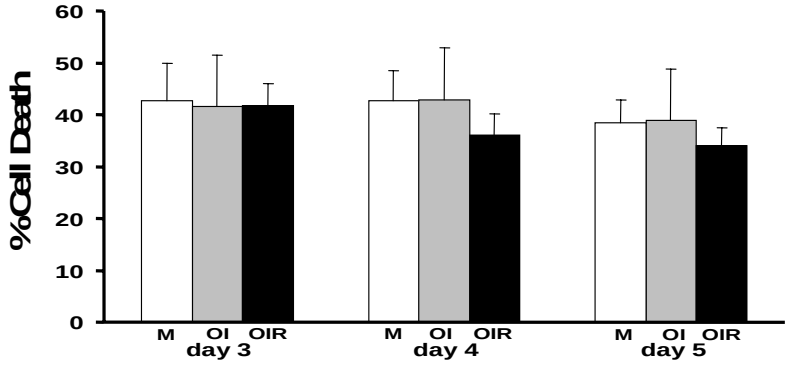


Figure 19B.
CD4+ T lymphocytes from eight different blood donors were either cultured in medium alone (M), or stimulated with OKT-3 and IL-2 in the absence (OI) or presence (OIR) of RA (100nM). The cells were analyzed for cell death by flowcytometri after three, four and five days, respectively. Mean percentages of dead cells ± SEMs are shown (*P<0.05, **P<0.01, paired samples t-test, n=8).

As shown in figure 19B, RA did not affect T cell viability at day 3, and caused only a minor, reduction in cell death at day 4 and 5. The minor effect of RA on apoptosis could therefore not explain the enhanced DNA synthesis shown in figure 1A.

4.1.7 RA stimulates the cell cycle machinery in T cells

One of the required steps for G1- to S - phase is the phosporylation of the pRB protein (Harbour and Dean, 2000a; Harbour and Dean, 2000b). Hyper-phosphorylated forms of pRB (ppRB) migrate slower in SDS-polyacrylamid gels, and can therefore be detected and distinguished from hypophosphorylated pRB (pRB). As shown in figure 20, pRB phosphorylation was strongly increased by RA. The effect of RA was first notable at day 2, and at day 3 pRB was primarily observed in its hyper-phosphorylated forms in the presence of RA. Phosphorylation of pRB is carried out by CDKs, whose activities are positively or negatively regulated by cyclins (of types D, E and A in the G1-S-phase) and CKIs (e.g.$p27^{Kip1}$ and $p21^{Cip1}$), respectively. T cells express cyclin D2, D3, E and A (Ajchenbaum et al., 1993; Firpo et al., 1994; Yam et al., 2002).As illustrated in figure 20, the protein levels of all the cyclins were clearly increased when the cells were stimulated with OKT-3 and IL-2. Whereas cyclin D2 expression was unaffected by RA, cyclin D3 levels was markedly enhanced by RA already at day 1, and at day 2 and 3 a strong up-regulation was observed. Cyclin E levels were unaffected by RA, while the cyclin A levels were upregulated by RA at day 2 and 3. It has been proposed that a down regulation of $p27^{Kip1}$ is a major target of IL-2-induced signaling (Firpo et al., 1994; Kwon et al., 1997; Nourse et al., 1994). This agrees with the observation that $p27^{Kip1}$ levels, in a time dependent manner, were reduced by treatment of T cells with OKT-3 and IL-2 (figure 20) A minor further reduction of $p27^{Kip1}$ in the presence of RA was noted at day 2, and a more apparent, but still relatively modest effect was observed at day 3 (figure 20). The expression of $p21^{Cip1}$ was unaffected by RA. In conclusion, the most striking effect of RA on the cell cycle machinery was its early induction of cyclin D3, followed by induction of pRB-phosphorylation.

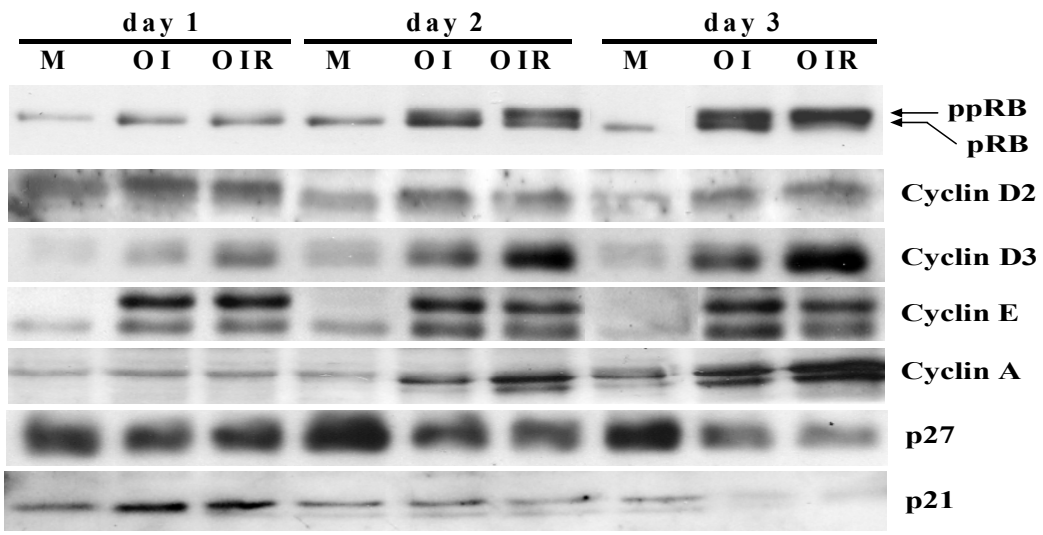


Figure 20. Effect of RA on pRB phosporylation, and expression of cell cycle regulatory proteins
Isolated CD4+ T lymphocytes were either cultured in medium alone (M), or stimulated with OKT-3 and IL-2 in the absence (OI) or presence (OIR) of RA (50nM). After 24, 48 and 72 hours, whole cell extracts were prepared, and Western blot analyses, using antibodies against the indicated proteins were performed as described in "Materials and Methods. Hypo- and hyper-phosphorylated pRB proteins are indicated (pRB and ppRB, respectively). One representative experiment of three is shown.

4.2 Mechanisms involved in RA-mediated potentiation of T cell proliferation

4.2.1 RA induces the expression of cyclin D3 at the level of mRNA

We next wished to investigate if the early induction of cyclin D3 induced by RA could be regulated at the mRNA level. Total RNA was isolated by the use of Qiagen RNeasy Mini kit, and analyzed by Northern blotting using ^{32}P-labeled cyclin D3 probes, followed by autoradiography. The results shown in figure 21, demonstrate a strong upregulation of cyclin D3 RNA-level in the presence of RA, indicating that RA enhances the expression of cyclin D3 at the mRNA level.

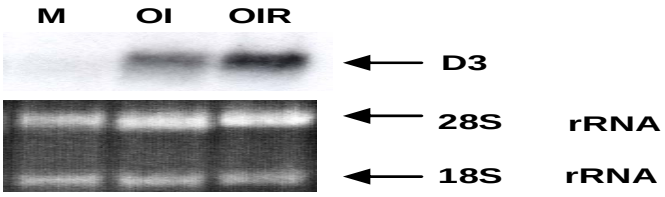


Figure 21. Effect of RA on cyclin D3 mRNA in CD4+ T lymphocytes.
CD4+ T lymphocytes were either cultured in medium (M) alone or with OKT-3 and IL-2 in the absence (OI) or in the presence (OIR) of RA (50nM). After 36 hours, total RNA was isolated and Northern analyses, using a specific radiolabeled probe to locate the RNA of interest, were performed as described in "Materials and Methods", and in collaboration with Nikolai Engedal. As a control of equal loading, the rRNA levels present in the samples are shown. One representative experiment of three is presented.

4.2.2 RA-mediated enhancement of T cell proliferation and cyclin D3 expression is dependent on IL-2-induced signaling

By using IL-2 receptor-saturating concentrations of IL-2 we had ruled out the possibility that RA stimulated the cell cycle machinery and T cell proliferation by increasing IL-2 production. Still, we could not exclude the possibility that RA mediated its effect by modulating TCR/CD3-, and not IL-2-induced signaling. To clarify which of these two signaling pathways that were affected by RA in our systems, we used a specific inhibitor of JAK, AG-490 (Kirken et al., 1999; Wang et al., 1999). To begin with we tested the effects of various concentrations of AG-490 on T cell proliferation induced by OKT-3 and IL-2. AG-490 was able to inhibit T cell proliferation in a dose-dependent manner, both in the absence and presence of RA (figure 22A), and RA did not affect cell viability (data not shown). We observed that the potency of RA to enhance T cell proliferation was gradually diminished with increasing concentrations of AG-490 (Figure 22A).Hence, whereas RA caused a 4-fold increase in 3H-thymidine uptake in the absence of AG-490, only a 2.9-1.8-, and 1.2-fold increase were found in the presence of the JAK-inhibitor at the following concentrations: 2.5μM, 10μM and 25μM. These results indicate that RA-induced T cell proliferation is dependent on JAK-mediated signaling from the IL-2R.

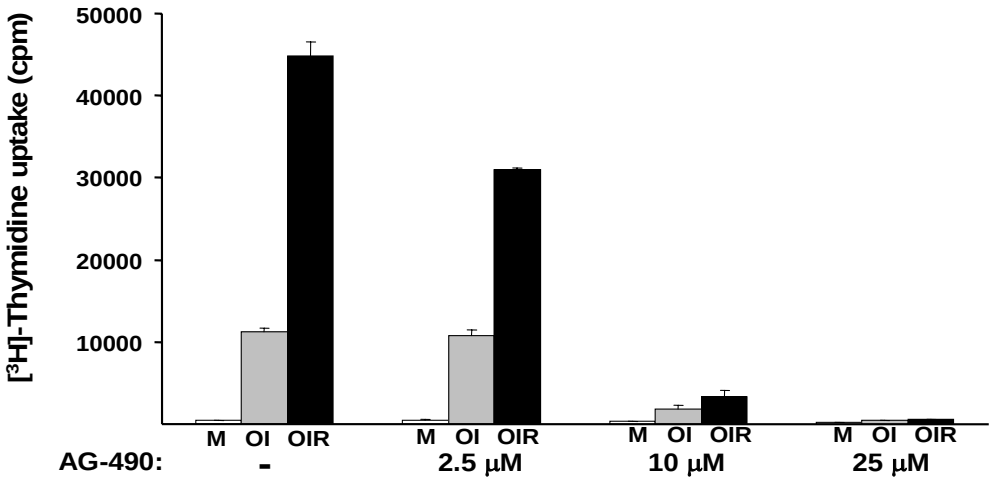


Figure 22A. AG-490 inhibits T cell proliferation and cyclin D3 expression.
CD4+ T lymphocytes were treated with various concentrations of AG-490 as indicated and either cultured in medium (M) alone, or stimulated with OKT-3 and IL-2 in the absence (OI) or presence (OIR) of RA (50nM). After 48 hours, cells were pulsed with ^{3}H-thymidine for 18 hours, as described in "Materials and Methods". Mean cpm-values ± standard deviations of triplicates from one representative experiment of three are shown.

Next, we assessed the effect of JAK-inhibition on the expression of cyclin D3. Remarkably, as shown in figure 22B, the expression of cyclin D3 was almost totally abolished by AG490 in cells treated with OKT-3 and IL-2, both in the presence and absence of RA. This indicated that IL-2 induced signals are crucial for the induction of cyclin D3 expression. To confirm that AG-490 indeed had inhibited JAK-activity, we investigated the inhibitor's effect on STAT3 tyrosine phosphorylation, which is known to be mediated by JAKs (Wang et al., 1999). As shown in figure 22B, OKT-3/IL-2 induced tyrosine phosphorylation of STAT3 was markedly inhibited by AG-490, both in the absence and presence of RA, indicating that AG-490 potently inhibited JAK-activity.

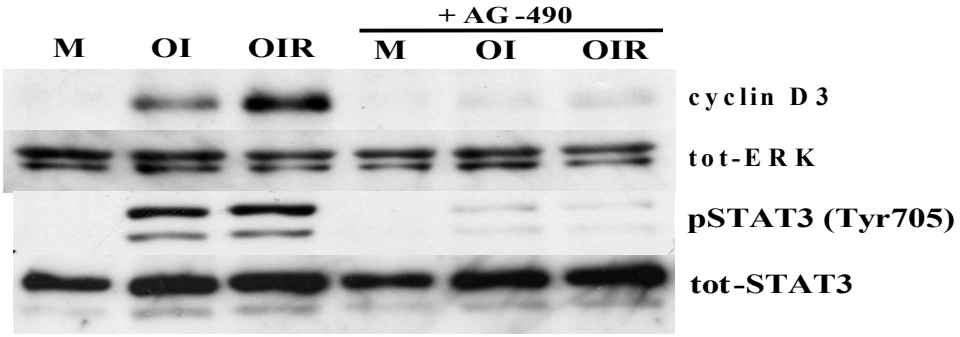


Figure 22B.
CD4+ T lymphocytes were either cultured in medium alone (M), or stimulated with OKT-3 and IL-2 in the absence (OI) or presence (OIR) of RA (50nM), as well as in the absence or presence of AG-490 (20μM), as indicated. After 72 hours, whole cell extracts were prepared and Western blot analysis, using antibodies against the indicated proteins were performed as described in "Materials and Methods". After immunoprobing with cyclin D3-or phospho-STAT3 specific antibodies, each membrane was stripped and re-probed with antibodies recognizing total ERK (tot-ERK) or total STAT3 (tot-STAT3) proteins, respectively, a control for equal loading. One reproduceable experiment of two is shown. The experiment was performed as described in Materials and Methods, and in collaboration with Nikolai Engedal.

4.2.3 The potentiating effect of RA on T cell proliferation seems not to be regulated by the ERK/MAPK pathway and the PI3K pathway downstream for the IL2-receptor

Both the ERK/MAPK pathway and the PI3K pathway downstream for the IL-2R play a role in cell proliferation and gene transcription. The signal molecule ERK of the ERK/MAPK pathway can regulate targets in the nucleus such as the transcription factor c-Myc (Pearson et al., 2001). C-Myc is known to be one of the genes regulated by retinoic acid (Balmer and Blomhoff, 2002). In the PI3K pathway, the protein-serine/threonine kinase Akt plays an important role in regulation of GSK-3, which regulates for instance D-type cyclins.

In order to investigate whether the ERK/MAPK pathway and/or the PI3K pathway, downstream for the IL-2 receptor are involved in the effect of RA on T cell proliferation, we used specific inhibitors. Thus we employed UO126, a specific blocker of the MAPK cascade by suppressing the activation of MAPK kinase (MKK1), and wortmannin, which inhibits PI3-kinase (Davies et al., 2000).

We first assessed the effect of wortmannin (0, 5μM) on DNA-synthesis. Wortmannin generally inhibited DNA synthesis induced by OKT3 and IL-2 (figure 23). When wortmannin was added after 24 hours, thus allowing the OKT3/IL-2 mediated signaling to occur; the effect of RA was not abolished (figure 23). These data indicate that RA-induced DNA synthesis is not dependent on the PI3K-pathway downstream of the IL-2R.

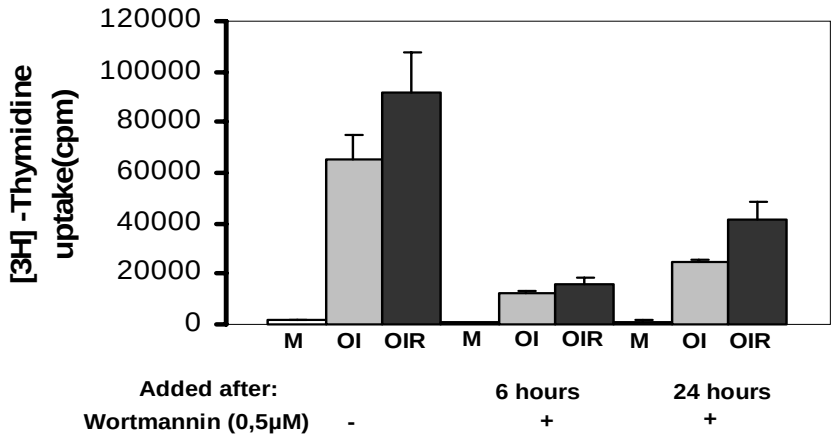


Figure 23. Wortmannin is not able to abolish the effect of RA on T cell proliferation. CD4+ T lymphocytes were either cultured in medium alone (M), or stimulated with OKT-3 and IL-2 in the absence (OI) or presence (OIR) of RA (50nM) or Wortmannin (0.5 μM). After 48 hours, cells were pulsed with ^{3}H-thymidine for 18 hours, as described in "Materials and Methods". Mean cpm-values ± standard deviations of triplicates from one representative experiment of three are shown.

We then investigated the effect of the MAPK inhibitor UO126 (10 μM) on DNA synthesis induced by OKT-3 and IL-2. As shown in figure 24 we observed that this inhibitor could not abolish RA's potentiating effect on T cell proliferation. When UO126

was added at time 0, it also inhibited the DNA synthesis induced by OKT3 and IL-2. These data suggest that RA-induced DNA synthesis is not dependent on the ERK/MAPK pathway.

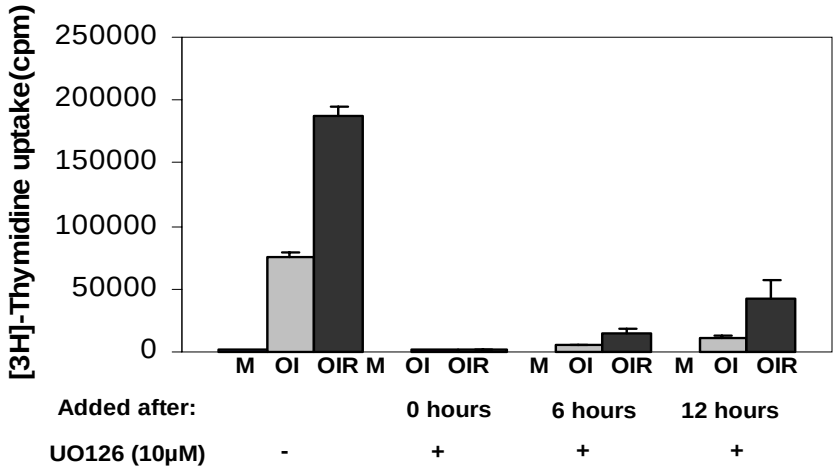


Figure 24. UO126 is not able to abolish the effect of RA on T cell proliferation.
CD4+ T lymphocytes were either cultured in medium alone (M), or stimulated with OKT-3 and IL-2 in the absence (OI) or presence (OIR) of RA (50nM) or UO126 (10 µM). After 48 hours, cells were pulsed with ^{3}H-thymidine for 18 hours, as described in "Materials and Methods". Mean cpm-values ± standard deviations of triplicates from one representative experiment of three are shown.

We also wished to assess the effect of PI3K- and MEKK1- inhibitors on the expression of cyclin D3. As shown in figure 25, OKT-3/IL-2-induced cyclin D3 expression was not abolished by wortmannin or UO126 in the presence of RA, which again indicates that neither the PI3K-pathway, nor the MAPK family pathway are involved in RA-stimulated T cell proliferation.

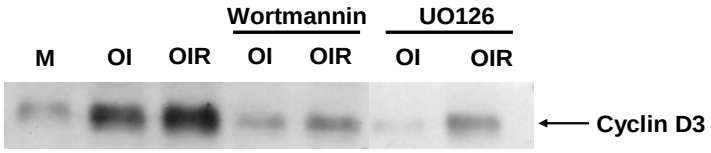


Figure 25. The effect of Wortmannin and UO126 on cyclin D3 expression.
CD4+T lymphocytes were either cultured in medium (M) alone, or stimulated with OKT-3 and IL-2 (OI) in the absence or presence (OIR) of RA (50nM). Wortmannin (0,5μM) or UO126 (10μM) were added about 10 hours after the start of the experiment. After 44 hours whole cell extracts were prepared and Western blot analysis, using antibodies against cyclin D3 were performed, as described in "Materials and Methods". One experiment of two is shown.

4.2.5 RA-mediated enhancement of T cell proliferation involves RAR

atRA is generally believed to mediate its cellular effects through binding to RAR and thereby regulate the transcriptional activity of target genes. Whereas atRA only binds to RAR, 9cisRA (9cRA) has high affinity to both RAR and RXR (Gronemeyer and Miturski, 2001). Previous work in our lab has shown that 9cRA and a specific RAR agonist, TTNBP, both increased IL-2 production to the same extent as did atRA in primary T lymphocytes stimulated with TPA (Ertesvag et al., 2002). As shown in figure 26, 9cRA, as well as the RAR-agonists TTNPB and AM-580, mimicked the effect atRA on increasing T cell proliferation. Interestingly, also the RXR-agonists, SR11217 (SR) and AGN194204 (AGN) were able to enhance OKT3/IL-2 induced DNA synthesis.

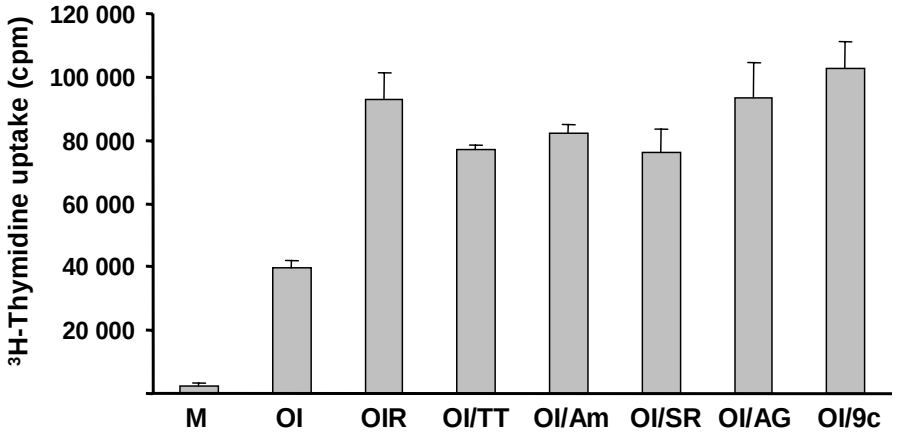


Figure 26. The effect of various RAR-and RXR-agonists on DNA synthesis.
CD4+ T lymphocytes were either cultured in medium alone (M) or stimulated with OKT-3 and IL-2 in the absence (OI) or presence of various RAR- and -RXR agonists at a final concentration of 100nM for all the agonists. OIR= OI+RA, OI/TT=OI+TTNPB, OI/Am=OI+Am580; OI/SR=OI+SR11217 and OI/AGN=OI+AGN194204, which are RXR-agonists. OI9c=OI+ 9 cis-RA. After 48 hours, cells were pulsed with ^{3}H-thymidine for 18 hours, as described in "Materials and Methods". Mean cpm-values of four independent experiments ± SEM are shown**.**

To verify the involvement of the RAR pathway, we used the RAR-selective antagonist, Ro 41-5253 (Apfel et al., 1992; Keidel et al., 1994). As shown in figure 27, a complete inhibition was noted at a concentration of 250nM of Ro 41-5253. Taken together, these results suggest that the effects of RA on cell proliferation are mediated through retionid acid receptor-dependent pathways.

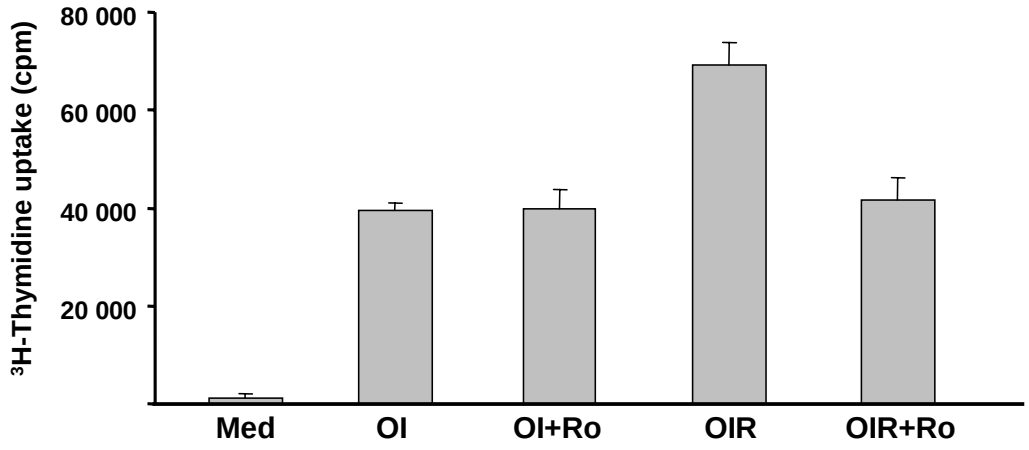


Figure 27. The effect of the RAR-antagonist Ro 41-5253 on DNA synthesis.

T cells were either cultured in medium alone (Med), or stimulated with OKT-3 and IL-2 in the absence (OI) or presence of RA (0.5 nM) (OIR) and/or Ro 41-5253 (Ro) (250nM). After 48 hours the cells were pulsed with ^{3}H-thymidine for 18 hours, as described in "Materials and Methods. Mean cpm-values ± SEMs from three independent experiments are shown.

5. DISCUSSION

5.1 Methodological considerations

5.1.1 Isolation of normal $CD4^+$ T- lymphocytes

We isolated $CD4^+$ T-lymphocytes from buffy coats obtained from human blood donors by first using density gradient separation. This method separates peripheral blood lymphocytes (PBL) from erythrocytes and granulocytes. Thereafter we employed "CD4 microbeads" to isolate quiescent $CD4^+$ T-lymphocytes by positive selection. As described by the manufacturer, Miltenyi Biotec, microbeads-binding antibodies against CD4, are developed for the separation of human cells based on the expression of CD4 antigen. CD4 is expressed on T helper cells, but also at a lower level on monocytes and dendritic cells. The positive selection method results in an extremely pure population of T cells with purity of at least 98 % resting, mature T cells. Less than 2% of the cells expressed CD8 (Cytotoxic T cells) or CD56 (NK cells), and the yield is satisfactory with about 150 million stained cells per buffy coat. The T cells are still quiescent after being isolated by positive CD4 selection. The advantage of using normal T lymphocytes is that these cells have normal physiology which is not always the case when working with malignant T cells, i.e. the human leukemia-derived T cell line Jurkat. This is an important aspect when the goal is elucidating mechanisms on how T cells work in combination with other agents in affecting the immune system. However, the disadvantage of using normal T-cells is that the isolation is time-consuming and expensive.

T cells can also be isolated by fluorescence activated cell sorting (FACS). The FACS-machine can separate the cells in a suspension on the basis of size and the color of their fluorescence. The cells of interest are labeled with specific monoclonal antibodies coupled to fluorescent dyes, and in case of T cell isolation one would use monoclonal antibodies against CD4. FACS is a good way of isolating lymphocytes based on their individual properties; however it is quite time consuming for isolation of large numbers of cells.

5.1.2 Activation of normal $CD4^+$ T-cells

In the body, T cells are activated by interaction between TCR and antigen displaced in the surface of antigen presenting cells. This, together with a second costimulatory signal, triggers a cascade of events that culminates in IL-2 production which drives the cell to proliferate. Activation of T lymphocytes in vitro can be mimicked by both synthetic and natural agents, such as anti-CD3 (OKT-3) and anti-CD28 antibodies, concanavalin A (Con A), phytohemagglutinin (PHA), and 12-O-tetradecaanoylphorbol 13-acetate (TPA) (Abb et al., 1979; Coligan et al., 1992). In our study we used the more physiological agent anti-CD3 antibody together with recombinant IL-2 (rIL-2) in order to activate the T cells. Anti-CD3, as its name imply, binds to CD3 which in the membrane of T cells is linked to TCR, and triggers the TCR/CD3 signaling events. RIL-2 binds to its IL-2 receptor and induces down-stream signaling resembling the in vivo process.

Others in our lab have used TPA or conA to stimulate T cells (Ertesvag et al., 2002). The tumor-promoting phorbol ester TPA activates T cells by mimicking diacylglyserol in activation of PKC (Nishizuka, 1984), and thereby bypasses the need for Ag-binding (Isakov and Altman, 1987). ConA, a mitogenic plant lectin, stimulates 20% or more of the T lymphocyte population (Kilpatrick, 1999). ConA binds to carbohydrate moieties of the T-cell receptor (TCR)-CD3 complex and co-stimulatory receptors, which is believed to induce a TCR- and co-stimulatory form of T-cell activation (Diehn et al., 2002). The stimulation of T cells with anti CD3/IL-2 is, however, considered to be a more "physiological" way of stimulating the cells.

5.2 Discussion of the results

5.2.1 The effect of RA on T cell proliferation and its role in stimulating the cell cycle machinery

Earlier studies in our lab had reported that RA enhances TCR/CD3-initiated signaling by increasing IL-2 production through a mechanism that involves RAR, and thus stimulates the proliferation of isolated human T cells (Ertesvag et al., 2002). Our goal now was to determine whether RA is able to modulate IL-2 induced signaling in T-lymphocytes. To

exclude the interference from RA inducing IL-2, we used saturatory concentrations of IL-2 in all the experiments. We could demonstrate that physiological concentrations of all-trans retinoic acid potently enhance DNA synthesis and the proliferation of freshly isolated human T lymphocytes that were simultaneously stimulated with anti-CD3 antibodies and saturating concentrations of IL-2.

i) The effect of serum on RA-mediated stimulation of T cells

In the present study, we have performed the experiments in the absence of fetal bovine serum (FBS). This was done in order to avoid retinol, which is found in serum (Fuchs and Green, 1981; Ishida et al., 2003). The serum we use has also been analyzed for the content of RA by the group of Rune Blomhoff, Department of Nutrition, and it was found that it contained approximately 0.6 μM retinol (data not shown). Experiments done by post doc Nikolai Engedal in our lab showed that the potentiating effect of RA on T cell proliferation was mostly abrogated in the presence of FBS. One explanation for this could be that some of the retinol which may be present in serum (Fuchs and Green, 1981; Ishida et al., 2003) is metabolized to RA by the T cells, thus providing enough RA to stimulate T cell proliferation in our experimental conditions. Another explanation of why serum diminished the effect of RA on T cell proliferation, could be that it contains factors (others than retinol) that may replace/ or inhibit the effect of RA. It has been shown that serum in fact contains factors that may stimulate (Clevenger et al., 1990) or inhibit, (Kucharz and Goodwin, 1988) IL-2 induced proliferation of T cells.

Culturing cells in the absence of serum can indeed be argued as being “physiological”, and also other groups have omitted serum from the culture medium (Allende et al., 1997; Friedman et al., 1993; Garbe et al., 1992). Naive lymphocytes are known to wander through the blood and the lymph to the secondary lymphoid organs, whereas the activated T cells may wander out to the infected tissue where they participate in the local “war” against the pathogen. The activation of T cells takes place in lymph nodes, spleen, Paye’s patches in the small intestine, in adenoid glands and the tonsils in the human body. In lymph nodes is probably the availability of growth factors from the blood restricted, due

to limited blood supply, and only low molecular substances are able to pass through. However, the blood flows right through the spleen, and therefore will the spleen be more exposed to growth factors. Thus, the amount of serum-derived growth factors available for T cells throughout the body may vary, and it is therefore important to examine the effect of RA also under limited inference from serum.

ii) RA affects IL-2 signaling per se and not TCR/CD3 signaling

We suggest that the stimulatory effect of RA on T cell proliferation under the present conditions is due to enhanced IL-2 mediated signaling, and not enhanced TCR/CD3-signaling. First, by using IL-2 receptor-saturated concentrations of IL-2, we ruled out a possible contribution from RA-mediated stimulation of IL-2 production. Second, in the presence of the JAK-inhibitor AG-490, which blocks IL-2 induced signaling, the observed increased T cell proliferation, was abolished. In addition, experiments performed by post doc Nikolai Engedal in our lab, revealed that the TCR/CD3-induced signaling was not blocked by the JAK-inhibitor, AG-490. TCR/CD3-induced signaling leads to a rapid upregulation of IL2R-α (CD25) on T cell surface, and he observed that the early induction of IL-2R-α was not affected by RA (data not shown). Our finding that RA may stimulate T cell proliferation by affecting IL-2 mediated signaling is supported by other studies. Sidell and coworkers studied IL-2-maintained lymphoblasts generated from human thymocytes (Sidell et al., 1993), whereas another research group studied the effect of RA on a murine IL-2-dependent T cell line, HT-2 (Jiang et al., 1992). Our group is however, the first to study normal human T lymphocytes, and we are also the first to study physiological activators like anti CD3, and to simultaneously stimulate the cells with anti CD3 and RA. This is relevant, since it has been shown that both IL-2 and IL-2R are expressed early in the activation of T cells (Lea, 2000).

iii) The effect of RA on the cell cycle machinery

IL-2 initiates T cell proliferation by promoting G1- to S-phase cell cycle transition (Nelson and Willerford, 1998). Thus, we wanted to investigate potential effects of RA on the important components of the cell cycle machinery which regulate the G1-to S-phase

transition in T cells. In accordance with the increased effect of RA on DNA-synthesis notable at day 3, we observed that the phosphorylation of pRB was strongly enhanced at day 2 and 3. Amongst the G1-cyclins, the earliest and most pronounced effect of RA was the induction of the cyclin D3 expression notable already at day 1. Cyclin D3 can contribute to succession through the cell cycle in two ways. First, cyclin D3 can activate CDK4 and CDK6 directly, and then this complex enters the nucleus and phosporylates the Rb-protein. Second, cyclin D3 can indirectly activate CDK2 through binding to CDK4 and CDK6, thus titrating the CKIs $p21^{Cip1}$ and $p27^{Kip1}$ away from the cyclin E/CDK2 complex, which further accelerates the cell cycle progression (Coqueret, 2002; McGowan, 2003). It has been reported that downregulation of $p27^{Kip1}$ expression is essential for IL-2-mediated CDK2-activation, and thus for G1-to S-phase in both human and murine T-cells (Firpo et al., 1994; Mohapatra et al., 2001; Nourse et al., 1994). In fact, we observed that RA moderately reduced the $p27^{Kip1}$ protein levels, but only at day 2 and day 3, and could therefore be a result, rather than the cause of G1- to S-phase transition. RA did not alter the $p21^{Cip1}$ protein levels, and we also observed that neither the expression of cyclin D2, nor cyclin E was affected by RA. Therefore, we suppose that RA allows CDK2 activation and pRb hyperphosphorylation predominantly by cyclin D3-CDK4/6 complex-mediated sequestration of $p21^{Cip1}$ and $p27^{Kip1}$. Furthermore, the cyclin A level was upregulated by RA. An interesting feature of cyclin A is that there are both an embryonic and a somatic form of the protein. Humans for instance have two A-type cylins, an embryonic specific cyclin A1 and a somatic cyclin A2. Cyclin A1 is only expressed in meiosis and very early embryos, whereas cyclin A2 is present in proliferating somatic cells (Yam et al., 2002).It is known that E2F is inhibited by binding to hypophosphorylated pRb family proteins during G1, but their phosphorylation by cyclin D/E-CDK complexes releases E2F, which is then able to activate the transcription of genes involved in S phase progression (including that of cyclin A). In addition, cyclins in G1 can stimulate the transcription of cyclin A (A2) in S phase (Yam et al., 2002). Thus, since cyclin A gene transcription is believed to be a downstream target of both cyclin D (Schulze et al., 1996)and pRb (Knudsen et al., 1999), the observed upregulation of cyclin A could as well be a direct consequence of RA-mediated enhancement of pRb-phosphorylation and increased cyclin D3 expression.

Previous studies have indicated that cyclin D2 (Lea et al., 2003), cyclin A and cyclin E (Firpo et al., 1994; Mohapatra et al., 2001)is mostly induced by TCR/CD3 signals, and only slightly induced by IL-2 initiated signals, whereas cyclin D3 expression (Lea et al., 2003; Modiano et al., 1995) seems to be primarily induced by IL-2 signaling. This is in agreement with our finding that cyclin D3 expression was almost fully abolished when IL-2 signaling was blocked by AG-490, both in the presence and absence of RA.

5.2.2 Mechanisms involved in RA-mediated potentiation of T cell proliferation

As cyclin D3 was the main cyclin affected by RA, observed by its early induction, we therefore focused on this protein. Cyclin D3 can be regulated at various levels. These include regulation of the transcriptional level of the gene (Wang et al., 1999), mRNA stability (Garcia-Gras et al., 2000), mRNA translation (Gutzkow et al., 2003) and cyclin D3 protein stability (Naderi et al., 2004). We observed that RA led to a very similar increase of both cyclin D3 protein and mRNA levels, indicating that the RA-enhanced expression of cyclin D3 was induced at the mRNA-level. Since, to our knowledge, there does not exist any RAR-responsive elements in the cyclin D3 promoter, we suppose that the effect of RA on cyclin D3 mRNA expression is indirect, but this remains to be elucidated.

In attempt to elucidate mechanisms involved in RA-mediated potentiation of T cell proliferation, we investigated if any of the known signaling pathways downstream for IL-2R are involved. JAK-mediated tyrosine phosphorylation of STAT5 is a proximal IL-2 induced signaling event which is involved in IL-2-mediated T - cell proliferation, and STAT3 requires both tyrosine and serine phosphorylation for full activation (Benczik and Gaffen, 2004; Ellery and Nicholls, 2002; Gesbert et al., 1998). Experiments performed by Nikolai Engedal showed that neither STAT-5 nor STAT-3 phosphorylation were enhanced by RA, but that their phosphorylation were both increased after 1 day of OKT3/IL-2-treatment (data not shown). STAT-5- phosphorylation was observed as early as 15 min after treatment with OKT-3 and IL-2 (data not shown). This suggests that

proximal IL-2 signaling is not dependent of STAT-5 or STAT-3 phosphorylation in our system. It has been demonstrated that the activation of STAT molecules does not appear to be essential for cellular proliferation (Ellery and Nicholls, 2002).

IL-2 R-ligation activates the PI3K/Akt-pathway and the MAPK/ERK pathway, which both are known to upregulate the expression of D-type cyclins (Ellery and Nicholls, 2002; Moon and Nelson, 2001; Sears and Nevins, 2002). We therefore investigated if these two pathways could be involved in the early upregulation of cyclin D3 expression caused by RA. However, by using wortmannin (a PI3K-inhibitor) and UO126 (a MKK1-inhibitor) we could exclude the involvement of these pathways. In addition we observed that none of these inhibitors could abolish the ability of RA to stimulate OKT-3/IL-2-induced DNA-synthesis. In support of this conclusion, Nikolai Engedal found that RA did not enhance cyclin D3 expression through Akt and ERK-stimulation. Nevertheless, he observed that OKT-3 and IL-2 induced a strong upregulation of both Akt and ERK which sustained for at least 22 hours (data not shown). Thus, from these data we have so far not been able to demonstrate that RA regulates a single, downstream IL-2-induced event, other than that it is dependent on JAK.

It is well known that retinoic acid mediates its effect mainly through the RAR/RXR-heterodimer, which binds to RA-response elements located in the promoter region of RA target genes (Balmer and Blomhoff, 2002; Despouy et al., 2003; Gronemeyer and Miturski, 2001). We wished to investigate if RA in our experiments did mediate its effect through RAR. In fact, we observed that two different RAR-agonists were able to mimic the effect of RA on T cell proliferation, and that a RAR-selective antagonist suppressed the effects of RA. Thus, it appears that RARs have a key role in mediating the effects of RA on transcriptional regulation. In previous studies done by our group, it was found that RA stimulated TCR/CDR initiated signals also through a mechanism involving RAR (Ertesvag et al., 2002). Interestingly, however, also the RXR-agonists, SR11217 (SR) and AGN194204 (AGN) were able to enhance OKT-3/IL-2 induced DNA synthesis. This is to our knowledge one of the very few studies having found any effect of RXR-agonists, and it could be due to some RA already being bound to RAR in the cells. This we suggest

in the light of previous studies where it has been reported that only RAR-, but not RXR-ligation is sufficient to activate RAR/RXR heterodimer, and in addition simultaneously ligation of both RAR and RXR acts synergistically on RAR/RXR transcriptional activity (Bastien and Rochette-Egly, 2004; Gronemeyer and Miturski, 2001).

As previously mentioned, we have not been able to demonstrate that RA regulates any of the known IL-2 signaling pathways downstream of the IL-2 receptor. However, it is likely that RA via the RAR/RXR acts further downstream, for example at the level of gene transcription, to change the expression of IL-2 responsive genes. Interestingly, experiments performed by post doc Nikolai Engedal, has recently shown that the protein expression of c-Myc, was upregulated within 5 hours after treating the cells with OKT-3 and IL-2. C-Myc is a transcription factor important in cell proliferation (Sears and Nevins, 2002). Cyclin D3 has recently been shown to be transcriptionally upregulated by c-Myc (Yu et al., 2005), and IL-2 induces c-Myc expression through a JAK3-dependent pathway (Nelson and Willerford, 1998). Thus, the observed RA-mediated up-regulation of c-Myc could give a possible explanation for the observed, JAK-dependent induction of cyclin D3 by RA. However, this still remains to be investigated.

Conclusions

The overall purpose of this thesis has been to elucidate if RA is able to enhance IL-2 mediated signaling in T cells, independent of its capacity to induce IL-2.

We have the following specific conclusions:

1. RA enhances IL-2 mediated proliferation in human T cells through a pathway dependent of JAK.
2. RA stimulates the cell cycle machinery induced by OKT3 and IL-2, and the most prominent effect of RA is the early induction of cyclin D3, as well as a strong phosphorylation of pRB.

3. RA induces the expression of cyclin D3 both at the mRNA-level and protein level. We were not able to show that RA affects any of the most known signaling pathways downstream of the IL-2 receptor (the JAK/ STAT-, the MAPK/ERK-, and the PI3K-pathway).
4. The effect of RA on IL-2 mediated signaling involves the nuclear receptor RARs and RXRs.

Taken together, our results indicate that RA enhances IL-2 mediated proliferation by inducing the expression of cyclin D3 in a JAK-dependent manner, and that the effects of RA are mediated via nuclear retionid receptors.

Future perspectives

We observed that RA led to a strong upregulation of cyclin D3 at the mRNA-level, and it would therefore be interesting to determine if cyclin D3 is a result of induced transcription or by stabilization of mRNA. This can be established by performing real-time PCR to measure the mRNA levels of cyclin D3, where the half life of cyclin D3 mRNA indicate its stability. Nuclear run-on experiments can be performed to analyze the transcriptional induction of cyclin D3, and finally it is possible to measure the stability of cyclin D3 mRNA by treating the cells by the RNA polymerase II inhibitor, DRB (5, 6-dichloro-1β-D-ribofuranosylbenzimidazole (Pallet et al., 2005).

We were not able to demonstrate an effect of RA on any of the common signaling pathways downstream of the IL-2 receptor, including the JAK/STAT-, the MAPK/ERK- and the PI3K-pathway. Flores and coworkers have demonstrated that IL-2 signaling also is dependent on the DAGK-α pathway by inducing phosphatidic acid production (Flores et al., 1999; Sanjuan et al., 2003). In their experiments, they employed a specific inhibitor of DAGK-α, R59949. We will use this inhibitor to unravel a possible effect of RA on this pathway.

Finally, we would also like to establish whether the RA-mediated upregulation of c-Myc is the mechanism behind the observed early induction of cyclin D3 by RA, and this will be explored by the use of siRNA against c-Myc.

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